DRIED SKIM MILK

HENRY

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Deterioration on

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368. DETERIORATION ON STORAGE OF DRIED SKIM MILK

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PART I. INTRODUCTION

BY KATHLEEN M. HENRY, S. K. KON, C. H. LEA AND J. C. D. WHITE

Henry, Kon & Rowland (1, 2) found, by the method of Mitchell (3, 4), that the biological value of the proteins of a bulk of dried skim milk stored at room temperature under conditions which did not exclude atmospheric moisture gradually deteriorated from 88·5 when the powder was a year and a half old to 71·1 three years later. The original moisture content of this powder and the rate of increase was not known, but at the end of the storage period it was 7%.

Findlay, Smith & Lea (5) had already observed that, with whole-milk powder, there was a critical moisture content below which fat deterioration was the first noticeable effect of storage, above which, however, severe non-fatty deterioration was the first to occur on keeping. These changes occurred very slowly at room temperature but much more rapidly at 37 and 47° C. The non-fatty deterioration resulted in unpleasant 'cardboard' or 'gluey' flavours, marked darkening of the powder and a great decrease in the solubility of the protein.

It was realized that the two observations were probably closely related, and a large-scale experiment was arranged by the three Research Institutes concerned to inquire fully into the deterioration on storage of skim-milk powder.

For this purpose a quantity of dried skim milk was prepared and samples of low, medium and high moisture content packed in air and in nitrogen were stored at three temperatures as described in Part II. At frequent intervals sample cans were removed for examination by taste and by various physical and chemical methods (Part III). On the basis of these tests a smaller number of the stored powders was selected for further examination for the nutritive value of the proteins by biological methods (Part IV), and for microbiological assay of 'essential' amino-acids (Part V). A brief discussion of the results obtained by the various methods is given in Part VI. A preliminary account of this work has already been published (6).

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PART II. PREPARATION, PACKING AND STORAGE OF THE EXPERIMENTAL POWDERS

By C. H. LEA and J. C. D. WHITE

(With 1 Figure)

Preparation (J. C. D. White)

For the purpose of the experiment, it was necessary to prepare three batches of spray-dried separated milk powder of at least 1 cwt. each and with moisture contents of the order of 2, 5 and 7%. A Gray-Jensen plant was used.

Bulk whole milk (late April 1945) was separated and dried in three 300 gal. batches. It was decided to use a pre-heating temperature of 165° F. (74° C.) with as short a holding time as possible. (For details of the pre-heating system in this type of drier see Hunziker (1).) In the present experiment, the first 300 gal. of separated milk were circulated from the storage tank through a tubular heater and back to the storage tank until the desired temperature of 165° F. was attained. This operation took about 20 min. 200 gal. were then pumped to the 'concentrator' or 'liquid collector', and thence to the drying-chamber. In the meantime, the remaining 100 gal., which subsequently passed to the 'concentrator' within about 15–20 min., were held at about 160° F. The batch was dried in about 45–60 min. Thus the milk which was dried towards the end of the 'run' was heated for a longer period than that dried at the beginning, but the average duration of the 165° F. pre-heating was about 30 min. The other two batches were treated in a similar way.

It was thought that powders of the requisite moisture contents could be obtained by making some very small alterations in the drying process, but when the drying of the powder was begun, it was found that the lowest moisture content conveniently obtainable was about 2·8% and the highest just under 5%.

One batch of milk was dried to give a powder of the 'low' moisture content, about 180 lb. being collected. The powder was well mixed and packed into cans holding 21 lb. The other two batches of separated milk were then dried to give powders with about 4·7% moisture. These two powders were mixed together very thoroughly in a stainless steel cheese vat to get a homogeneous powder with a uniform moisture content. Half of the mixture was packed into 21 lb. cans and the moisture content of the other half was raised from 4·7 to about 7–8% in the following way.

About 190 lb. of the powder were spread out on two curd-cooling troughs made of tinned steel and having a depth of 9 in. and a combined surface area of about 33 sq.ft. The air of the room was kept humid by water vapour from an open vat of water kept boiling by a steam jet. Every 10–15 min., the powder was thoroughly mixed and built into ridges to increase the surface exposed. On the first day, the powder was exposed for 12 hr., the relative humidity being about 75% for most of the time but rising to 85% towards the end of the period. Representative samples were taken at intervals for the determination of moisture content by heating in an air oven for 3 hr. at 101·5° C. The powder was then filled into cans for the night. Next morning the apparent moisture content of the powder had fallen from 6·2 to 5·8% despite the fact that it had been in tightly closed cans in the interval. This decrease may have been due to crystallization of a small proportion of the anhydrous lactose (cf. below and Part III, p. 303). On the second day, rain fell heavily and the R.H. remained fairly constant at 95%. After 8 hr. exposure with frequent thorough stirring as before, the moisture content had risen to just

over 7%. Finally, the powder was thoroughly mixed and packed into 21 lb. cans. Fig. 1 illustrates the rate of increase of moisture content.

Thus, there were now available three batches of separated milk powder of low, medium and high moisture content, which will be denoted by the letters 'L', 'M' and 'H' respectively. 'L' was a type of powder which can readily be produced on a normal spray-drying plant when the necessary precautions are taken for obtaining a powder of low moisture content, 'M' was a powder of the kind frequently obtained under usual factory conditions, while 'H' had a moisture content as encountered in practice when powder has been stored under unsuitable conditions.

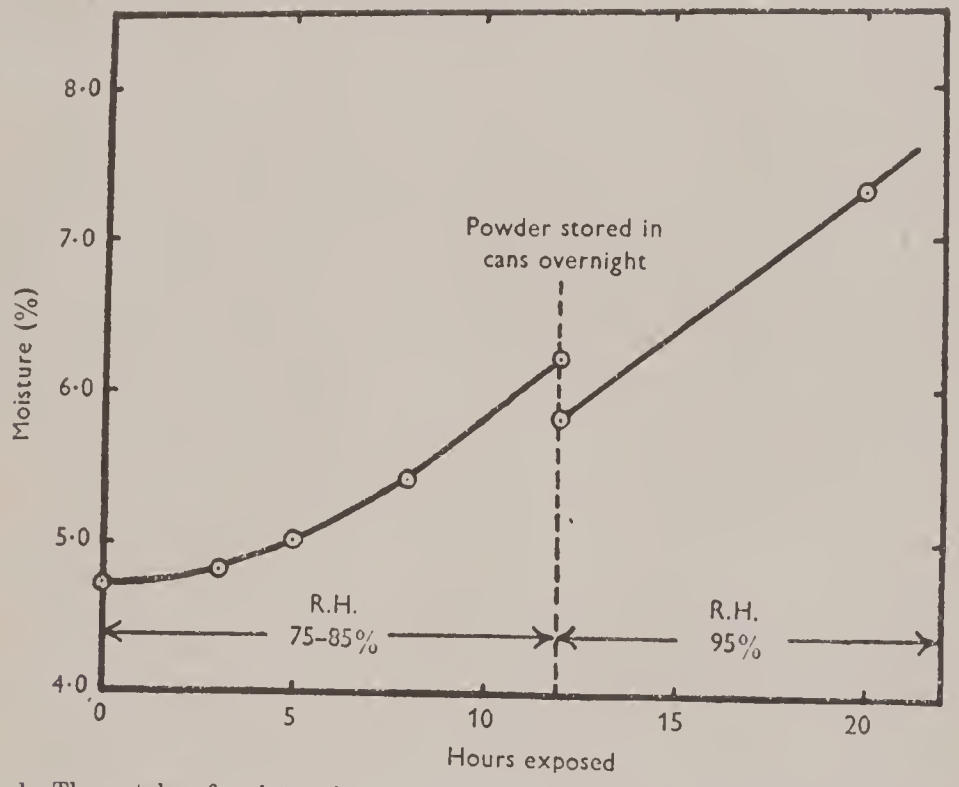

Fig. 1. The uptake of moisture by powder of 4·7% moisture content during conversion to powder of 7·3% moisture content.

Bacteriological examination of the powders by Dr C. Higginbottom indicated that no increase in bacterial population occurred during the raising of the moisture content. The technique of examination has been described previously (2). The plate counts are given in Table 1.

Table 1. *The bacterial count of the fresh powders after packing*

Powder	Plate count/g. powder	
	3 days at 37° C.	5 days at 30° C.
H	132,500	239,500
M	180,000	281,000
L	186,000	290,000

COMPOSITION AND MOISTURE CONTENT

The powders were analysed for fat by a modified Röse-Gottlieb method (3), and for protein by the usual Kjeldahl procedure. Lactose was measured polarimetrically after precipitation of the protein by mercuric nitrate or cadmium sulphate (4, 5). The analyses, which are given in Table 2, are typical of normal dried skim milks, except, of course, for the unusually high moisture content of H powder.

Moisture contents, determined by heating in an air oven for 3 hr. at 101·5° C., are given in Table 2. It is known, however, that the apparent moisture content of milk powder varies in some degree with the conditions of the determination. This variation will be particularly marked when any appreciable proportion of hydrated lactose is present, since α-lactose monohydrate, which loses very little of its 5·0% of moisture during 3 hr. at 100° C. in the air oven, is dehydrated completely in 20 hr. at 100° C. and 5 cm. pressure (6).

Table 2. *Analysis of the fresh powders*

	Powder			Moisture-free basis		
	H	M	L	H	M	L
Moisture*	7·3	4·7	2·9	0·0	0·0	0·0
Fat	1·6	1·5	0·8	1·7	1·6	0·8
Protein (N × 6·38)	32·0	32·7	34·2	34·5	34·3	35·2
Lactose† etc. (by difference)	50·9	52·9	54·2	54·9	55·5	55·9
Ash	8·2	8·2	7·9	8·9	8·6	8·1

* 3 hr. at 101·5° C.

† Lactose (anhydrous) estimated polarimetrically was 50·6, 52·8 and 53·6% for H, M and L powders respectively.

Table 3. *Moisture content of the experimental powders as determined under various conditions*

Powder	Sample no.	Hours at 100° C. (air oven)	Hours at 100° C. and 3 cm. pressure (vacuum oven)			
		3	5	10	15	20
H	1	—	7·5	7·6	7·5	—
	2	—	7·8	7·8	7·8	—
	3	—	7·7	—	—	—
	4	7·2	7·4	7·5	7·5	7·5
	5	7·2	7·4	7·6	7·6	7·6
	6	7·4	7·6	7·7	7·8	7·8
	7	—	7·4	7·4	7·5	7·5
	8	—	7·5	7·5	7·6	7·6
	Average	7·3	7·5	7·6	7·6	7·6
M	1	—	4·9	—	—	—
	2	4·7	4·9	5·0	5·0	5·0
L	1	—	2·9	—	—	—
	2	2·8	2·9	3·0	3·0	3·0

Apparent moisture contents were therefore determined on several samples of the powder after heating for 3 hr. in the air oven, or for periods up to 20 hr. in the vacuum oven at 100° C. (Table 3). Although the results do not exclude the possibility that a small proportion of the lactose had already crystallized during preparation of the powder, the greater part of the lactose must have been in the form of a non-crystalline, supercooled 'glass', such as is usually present in spray-dried milk or whey powders (7). There is a suggestion of a slight heterogeneity in the moisture figures for H powder, which will be referred to again in connexion with certain of the chemical results. Supplee (8) and Lampitt & Bushill (9) have previously stressed the difficulty of raising uniformly the moisture content of a bulk of milk powder. The equilibrium relative humidities of the three powders are given in Part III (p. 304).

Packing in air and in nitrogen

Part of each of the three powders was packed, at the Hannah Institute, in A 1 (315 ml.) tinplate 'open-top' cans, 150 g. per can, and sealed in air. Another portion of each powder was weighed into similar cans and gas-packed at Cambridge, using oxygen-free

nitrogen and a cabinet of the type previously deseribed (10). The remainder of the powders, also gas-paeked, was held at −20° C. in reserve. The seams of all eans were treated with bitumen to ensure gas tightness.

All three powders proved to be of the very rapidly desorbing type, the amount of 'entrapped' oxygen being eomparatively small, and 'desorption' was practically complete within 24 hr. (Table 4). Some slight diffieulty was experieneed in gas-paeking owing to

Table 4. *Desorption of oxygen from the powders after gas-packing*

	% oxygen in the head-space gas after hours			
Powder	0	3	24	72
H	0·1	—	1·1	1·3
M	0·1	—	1·2	1·3
L	0·1	0·9	1·6	1·6

the unusually high permeability of the partieles, whieh permitted partial exhaustion of air from the eavities during the exhaust eycle with a eonsequent tendeney for air to be drawn into the eans during the brief interval between removal from the eabinet and soldering of the brogue holes. This effeet was minimized by holding the powder for 15 min. under a positive pressure of nitrogen before opening the eabinet. Analysis of the head-spaee gas in a number of the eans after gassing three times at intervals of one day showed a eontent of oxygen ranging from 0·0 to 0·3% with an average of 0·15%.

Conditions of storage

Air- and gas-paeked eans of all three powders were stored at both laboratories, the storage temperatures aimed at being 20·0, 28·5 and 37° C. At the Hannah Institute the cans were stored in ineubators, the temperatures of whieh were eheeked daily. At Cambridge thermostatieally-eontrolled rooms with rapid air eirculation were used, open spacing during the first few days ensuring that the powders attained the required temperature within a very few hours. The temperatures, reeorded by thermometers moved among the eans, were read daily and, over the whole period averaged well within 0·1° of the required figure, with a standard deviation from this mean of about 0·1° C. The storage experiment was commeneed at the same date in both laboratories, and the powders examined thereafter at intervals as deseribed in Parts III, IV and V.

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PART III. PHYSICAL, CHEMICAL AND PALATABILITY CHANGES IN THE STORED POWDERS

By C. H. LEA and J. C. D. WHITE

(With 22 Figures)

As already described in Part II separated milk powders of three moisture contents, packed in air and in nitrogen, were stored at three temperatures at both the Low Temperature Research Station and the Hannah Dairy Research Institute, and sample cans removed at intervals for examination by taste and by various physical and chemical methods. At a comparatively early stage of the work it became apparent that appreciably different rates of deterioration of the least stable powder at the highest storage temperature were being observed at the two laboratories, differences which were probably due to minor variations in effective storage temperature coupled with a very high temperature coefficient of some of the reactions involved in deterioration. Therefore, as a precautionary measure, some of the chemical determinations were duplicated at the two laboratories. In such cases one set only of the results has been presented in detail, the other being given in summarized form.

Changes in palatability

The necessity for carrying out tasting tests in experimental work on the keeping properties of milk powder, and some of the weaknesses inherent in the tasting panel technique have previously been discussed in connexion with whole-milk powder(1). In order to obtain evidence as to the magnitude of these difficulties, and at the same time to reduce their effect, the system devised for use with whole-milk powder was extended to separated powder. According to this scheme the various series of deteriorated powders were examined independently at the two laboratories, each choosing its own technique, and a comparison of the results was made only at the end of the storage experiment.

Technique

In the method used at Cambridge the stored powders were reconstituted in distilled water at 20° C. in proportions corresponding to 10 g. dry milk solids per 100 ml. water. The samples, labelled in a code unknown to the tasters, were graded half an hour later by a panel of six persons, marks being awarded according to the scale: 6 = very good, 5 = good, 4 = fairly good, 3 = fair, 2 = rather poor, 1 = poor, 0 = very poor, as previously used for full-cream powders(1, 2). While the attainment on this scale of a score of 4 is usually considered to mark the end of the life of a powder as a product of reasonably good quality for household use, no large-scale consumer trials were carried out and the function of the numerical scale employed in these experiments was mainly to facilitate comparison between the various treatments. The standard adopted was fairly severe; the powders scoring 3 or even 2 would probably be quite usable for some manufacturing or domestic cooking purposes.

In the method used at the Hannah Institute the powders were coded, reconstituted in distilled water at 35° C. using a powder to water ratio of 1 : 10, and tasted within 30–60 min. by a panel of five persons. A powder of good quality was included in each tasting as a standard or control powder. The tasting and scoring technique has been described previously with reference to whole-milk powders(1, 2), but the scoring system is briefly as follows: 0 = very good and very like ordinary fresh milk, 1 = fairly good and

quite palatable, 2 = slightly but definitely unpalatable due to the presence of slight 'off'-flavours, 3 = unpalatable due to the presence of pronounced 'off'-flavours, 4 = very unpalatable. This method of scoring measures the '*un*palatability' of a powder and the average of the 'off'-flavour marks awarded to it by the five tasters is termed its 'off'-flavour score. When a powder passes 'off'-flavour score 1·0, the trained panel is beginning to detect the development of slight 'off'-flavours. When score 2·0 is reached, the 'off'-flavour would probably be noticed and objected to by the ordinary consumer.

H, M and L powders tasted prior to storage against separated fresh milk (flavour score 6·0, 'off'-flavour score 0·0) were awarded scores of 5·2, 5·6 and 5·5, and 0·4, 0·2 and 0·1 respectively according to the two systems. It was obvious that H powder had already commenced to deteriorate in flavour during the short interval of approximately 10 days occupied by rail transport, etc., between preparation and beginning of the storage

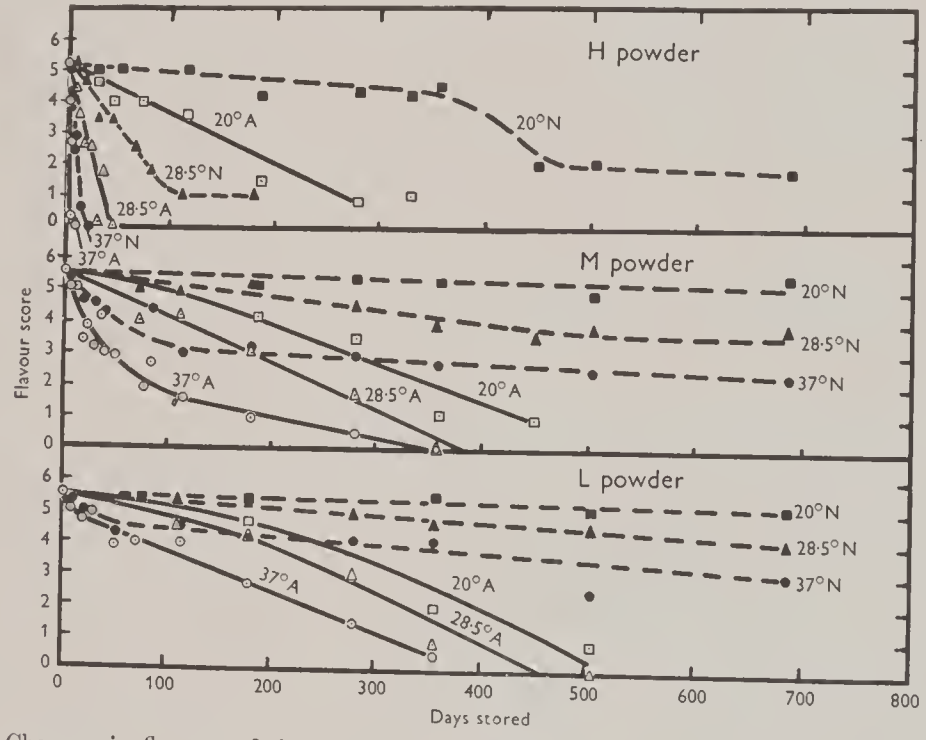

Fig. 1. Changes in flavour of the air-packed and nitrogen-packed powders during storage at 37, 28·5 and 20° C. (Cambridge results).

experiment. Thereafter only M or L powder, gas-packed and held at −20° or at 0° C. was used as control in the grading of powders stored at higher temperatures.

The results of the storage experiments, which are given in Figs. 1 and 2, and in Table 1, may be summarized as follows.

Effect of moisture content

The most noteworthy feature of the results was the great rapidity of deterioration of H powder, as compared with M and L powders. Storage life, as determined by measurement of palatability, ranged from about 2 days for H powder packed in air and stored at 37° C. to periods of the order of 2 years or longer for powders of low and medium moisture content packed in nitrogen and stored at 20° C.

The higher the storage temperature the greater was the deleterious effect of a high moisture content. In the air-stored series the ratios of the storage lives of the H, M and L powders, as estimated by the Cambridge panel from the average of the data for flavour

scores 4, 3 and 2, were of the order of 1 : 16 : 50 at 37° C., 1 : 10 : 15 at 28·5° C. and only 1 : 2·1 : 2·6 at 20° C. (cf. Table 1). The corresponding ratios as estimated by the Hannah Institute panel were even higher, 1 : 58 : 87 at 37° C., 1 : 21 : 30 at 28·5° C. and 1 : 3·5 : 4·1 at 20° C. Data for the gas-stored powders are incomplete.

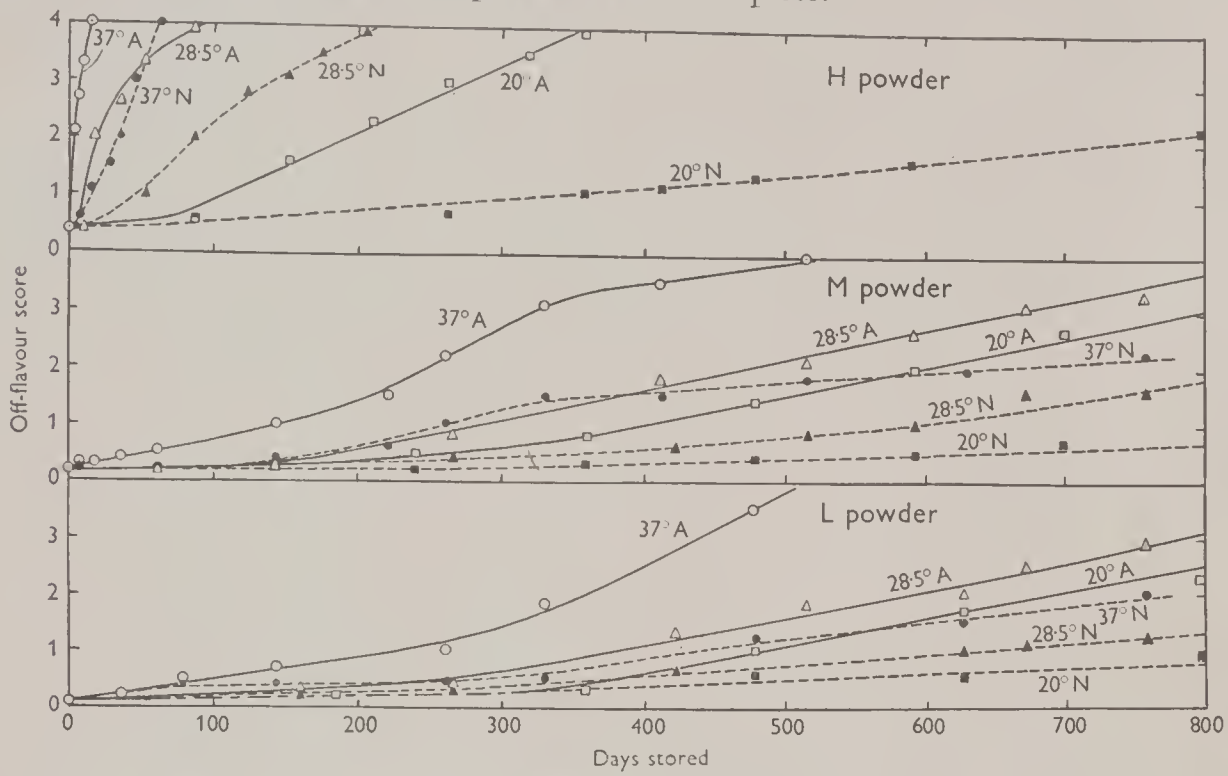

Fig. 2. Changes in flavour of the air-packed and nitrogen-packed powders during storage at 37, 28·5 and 20° C. (Hannah Institute results).

Table 1. *Deterioration in palatability of skim-milk powder on storage**

		Cambridge results: Time taken to deteriorate to flavour score (days)						Hannah Institute results: Time taken to deteriorate to 'off'-flavour score (days)					
		Air-pack			Nitrogen-pack			Air-pack			Nitrogen-pack		
Powder	Storage temp. (° C.)	4	3	2	4	3	2	1	2	3	1	2	3
H	37	2	3	4	5	8	10	2	4	8	18	34	49
	28·5	10	18	27	30	55	78	12	21	44	51	92	140
	20	75	140	200	380	430	480	115	180	270	340	*c.* 690	—
M	37	20	44	88	50	200	—	145	250	320	260	*c.* 660	—
	28·5	110	180	250	400	—	—	290	480	*c.* 660	590	—	—
	20	190	270	360	—	—	—	410	590	—	—	—	—
L	37	80	160	240	250	700	—	240	350	430	450	—	—
	28·5	190	270	330	—	—	—	370	590	—	*c.* 650	—	—
	20	250	330	400	—	—	—	500	*c.* 670	—	—	—	—

* Absence of a figure in the table signifies a period of over 700 days.

Temperature coefficients

The temperature coefficient of the reactions responsible for deterioration of flavour in H powder was unusually high, an increase in rate of spoilage of the order of sixfold or more resulting from an increase in storage temperature from 20 to 28·5° C., or from 28·5 to 37° C. This was the case for the Cambridge results whether storage was in air or in inert gas. A similar factor was found by the Hannah Institute panel for air-stored powder over both temperature ranges, and for gas-stored powder over the lower range between 28·5 and 37° C. in nitrogen a rather lower factor of approximately 3 was obtained

The effect of temperature on the deterioration of M and L powders was very much less marked. For M powder the acceleration factor averaged only 1·5 for both panels over the range 20–28·5° C., and 2·0 (Hannah Institute) or of the order of 5 (Cambridge) for the range 28·5–37° C. For L powder the corresponding factors averaged only 1·2 and 1·6 for the two ranges.

It may be concluded that in powder of high moisture content, and perhaps even to some extent in powder of medium moisture content at high storage temperatures, changes resulting in spoilage of flavour occur which do not take place to any appreciable extent in powder of low moisture content even at 37° C. The nature of these changes will be discussed later (p. 335).

Effect of gas-packing

Packing in an atmosphere of nitrogen considerably delayed deterioration in flavour, the average 'protection factor' for all powders, storage temperatures and degrees of 'off'-flavour, as estimated by the Cambridge panel, being about 3. The corresponding factors recorded by the Hannah Institute panel were of the order of 2 for L and M powders, 3 for H powder at 20 and 28·5° C., and as much as 8 for H powder at 37° C. (Table 1). The advantages of nitrogen-packing, which can be seen more clearly in Figs. 1 and 2, suggest that in powders of all moisture contents part at least of the 'off'-flavours produced must result directly or indirectly from reactions involving atmospheric oxygen.

Types of 'off'-flavour

Two main types of 'off'-flavour were detected. In the air-packed series, particularly at high moisture content, the powders commenced by developing a 'stale' or 'cardboard' flavour which gradually intensified to a characteristic nauseating and very unpleasant 'gluey' taste. In air-packed powders of low moisture content 'off'-flavours developed very much less rapidly and were less obviously 'glue-like' in character. Since a recognizably tallowy flavour has previously been observed to develop in air-packed separated milk powder containing 2·4% of fat (3), it is quite possible that oxidation of the rather smaller proportions of residual fat present in these powders (cf. Part II, Table 2) may have made a significant contribution to deterioration over long periods of storage, although a tallowy flavour could not be identified specifically.

The nitrogen-packed powders did not develop the same type or degree of unpalatability on storage. Their typical 'off'-flavour tended to be a 'heated', 'cooked', 'caramelized' or slightly 'stale' flavour, suggestive of evaporated milk. A long-stored, gas-packed high moisture powder might have a very poor solubility, a brownish colour, and show an abnormal degree of frothing* on reconstitution, yet, its predominantly 'caramelized' flavour, though far removed from that of fresh milk, could not be considered really unpleasant. Gas-packed powders of low moisture content showed very little deterioration in flavour during the period of the experiment.

Since the flavour of air- and nitrogen-packed powders so frequently differed markedly in quality as well as in degree, it was inevitable that different groups of observers would differ in their estimates of the magnitude of the improvement in palatability or in storage life effected by packing in inert gas. A sufficient measure of agreement was, however,

* Such frothing is known to be a property of the 'melanoidins' formed by the interaction of amino-acids or protein and reducing sugars.

registered between the two panels to establish the usefulness of gas-packing for preserving the palatability of separated milk powder, as had previously been done for whole-milk powder (3).

Changes in colour

For the measurement of colour, which was carried out at both laboratories, the powders were packed in a standardized manner into small porcelain dishes and examined in the Lovibond Tintometer with artificial light attachment. At Cambridge, the Lovibond-Schofield modification of the instrument was used, in which illumination was by C.I.E. Standard Illuminant B, consisting of gas-filled metal filament bulbs operating at a colour temperature of 2848° K., used in combination with the specified colour filter solutions.

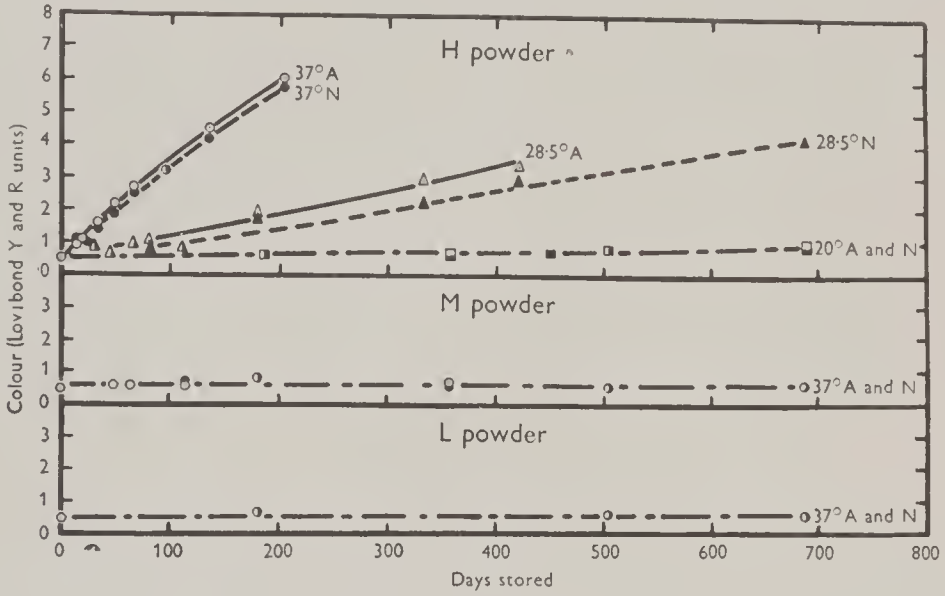

Fig. 3. Development of discoloration in the stored powders (Cambridge results).

Table 2. *Rates of discoloration of skim-milk powder during storage*

Powder	Storage temp. (° C.)	Increase of discoloration (Lovibond units/100 days)* Cambridge results Air-pack	Cambridge results Nitrogen-pack	Hannah Institute results Air-pack	Hannah Institute results Nitrogen-pack
H	37	3·0	2·8	2·6	2·2
	28·5	0·7	0·6	0·7	0·6
	20	0·0 (7)	0·0 (6)	0·0 (5)	0·0 (3)
M	37	0·0 (2)	0·0 (2)	0·0 (3)	0·0 (3)
L	37	0·0 (0)	0·0 (0)	0·0 (2)	0·0 (2)

Colour quality in both instruments was matched by standard yellow and red glass slides, while the relative brightness of the sample and of the comparison field could be equalized by means of the obturator vane fitted to the light cabinet, or by the use of neutral tint slides. In practice it was found that little use of these latter controls was necessary over the limited range of brightness encountered in the storage experiments, and, for simplicity, colours have been recorded simply as the sum of the yellow and red units used.

Reproducibility in the measurement of colour by this means was not of a very high order, and different operators were liable to obtain appreciably different results. The figures obtained, however, indicate sufficiently clearly the order of magnitude of the effects of the various factors under investigation on the rate of discoloration of separated milk powders (Fig. 3, Table 2).

As with the majority of the other characteristics measured, high moisture content and high storage temperature were the factors chiefly concerned in causing spoilage. The progress of discoloration was very roughly linear, the air-packed powders changing just perceptibly more rapidly than those in nitrogen. Relative rates of discoloration of H powder at 20, 28·5 and 37° C. were of the order of 1 : 13 : 53 (Table 2). The very high temperature coefficient between 20° and 28·5° C. and the slight induction period visible at the latter temperature are probably due, in part at least, to an increasingly delayed crystallization of lactose as the storage temperature is reduced (cf. p. 305).

Changes in the colour of M and L powders, even after 2 years at 37° C., were very slight.

CHANGES IN pH (J. C. D. WHITE)

The powders were reconstituted in distilled water to give a 9% solution of milk solids, and the hydrogen-ion concentration measured by glass electrode and pH meter. The results (Fig. 4) show that, as with the development of colour, high moisture content and

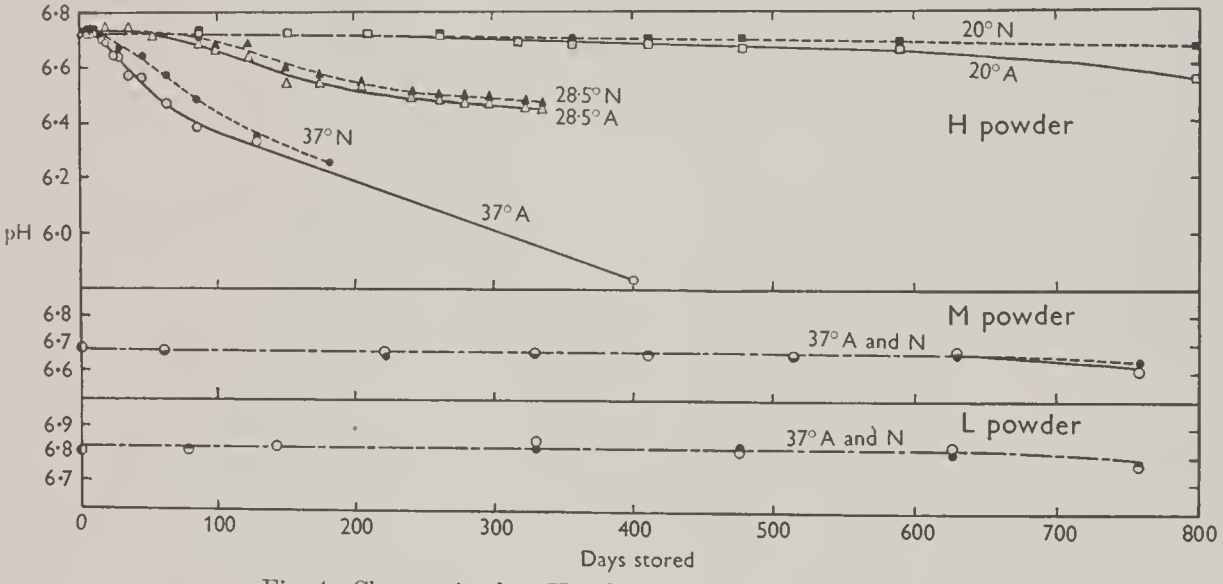

Fig. 4. Changes in the pH value of the powders on storage.

high storage temperature were the factors mainly concerned in producing a change in pH: after 400 days at 37° C. the pH of reconstituted H powder had fallen from 6·72 to 5·83. Packing in nitrogen slightly retarded, but did not prevent, the decrease. M and L powders retained their initial pH values almost unchanged, even after storage for 2 years at 37° C.

Further data on pH changes in H powder, and evidence bearing on the cause are given in a subsequent section (p. 318).

CRYSTALLIZATION OF LACTOSE, AND CHANGES IN EQUILIBRIUM RELATIVE HUMIDITY (C. H. LEA)

Lactose exists in spray-dried milk powder as a supercooled, highly concentrated solution or 'glass' composed of α- and β-sugars in the ratio of about 1 : 1·5, which is stable for long periods at normal temperatures and low relative humidities. If the moisture content of the powder is sufficiently high, or if a dry powder is permitted to absorb sufficient water vapour from the atmosphere, α-lactose monohydrate, which contains 5·0% of water, crystallizes out on storage, and the powder 'cakes', the change being greatly

accelerated by high storage temperatures. At sufficiently high relative humidities (e.g. 70%) β-lactose is slowly converted to the crystalline α-hydrate (4).

In the present experiments H powder, after storage at 37° C. for a few days, set to a solid mass in the can, indicating extensive crystallization of the lactose. This conclusion was confirmed by observation under the polarizing microscope, and by seeding tests with a supersaturated lactose solution (4). It was not, however, found practicable to make either of these tests quantitative.

Since it was felt that the influence of moisture on chemical or physical changes occurring in the powder during storage would be more closely related to the activity of water as indicated by relative humidity measurements than to moisture content as determined by one or other of the empirical methods available, equilibrium relative humidity measurements were carried out as follows.* Several 1 g. portions of each sample of powder were weighed into light aluminium dishes 2 in. in diameter, and each dish was suspended over a solution of sulphuric acid of suitable concentration in a closed, 'Kilner' type glass jar. The wire supporting the dish passed between the two halves of a small, split rubber stopper inserted in a hole in the lid of the jar and terminated in a loop for attachment to the arm of a balance. With this arrangement each sample of powder could be weighed at intervals without removal from its particular atmosphere. The jars were stored and the samples weighed in a thermostatically controlled room at 20° C. The relative humidity at which the sample neither gained nor lost weight, as obtained from a plot of the gains and losses at the various relative humidities used, was taken as the equilibrium R.H. The determination was not considered satisfactory unless sulphuric acid solutions within 5% above and 5% below the equilibrium value for the powder under examination had been included in the series. It should be noted that, for convenience, equilibrium R.H. values were determined at 20° C. for all milk powder samples, irrespective of the temperature at which they had been stored. Values for the fresh powders were: H powder, 7·5–7·8% moisture, 41–43% R.H.; M powder, 5·0% moisture, 29% R.H.; L powder, 3·0% moisture, 17·5% R.H. There was again a suggestion of slight heterogeneity in H powder.

In Fig. 5, equilibrium R.H. values for the various stored powders are plotted against storage time, each point being representative of the contents of an individual can. In order to examine more closely the first portion of the curve for H powder at 37° C., 5 g. portions of control powder from the well-mixed contents of a single can which had been held under nitrogen at −20° C. were sealed in glass tubes, heated rapidly to 37° C. in a water-bath, and stored at 37° C. The superior reproducibility and slightly higher equilibrium value after crystallization obtained under these conditions again suggest a slight lack of homogeneity in the large bulk of H powder used in the main experiment.

Crystallization of all the α-lactose originally present in the supercooled 'glass' form would absorb only about 1·1% of the 7·5–7·8% of moisture originally present in H powder. Lactose as concentrated syrup or 'glass' is much more hygroscopic than when crystalline. Crystallization of α-lactose hydrate would therefore cause dilution of the remaining lactose glass (some 60% of the original total) and tend to cause an increase in equilibrium R.H. The observed increase in this value from *c.* 42 to *c.* 55% on storage of H powder can therefore be explained qualitatively by the crystallization of lactose.

After a relative humidity of about 55% had been attained there appeared to be no further increase on continued storage despite the fact that a gradual conversion of β-

* The method described is an adaptation of that used by Gane (*J. Soc. chem. Ind., Lond.* (1941), **60**, 44) for investigation of the water relations of wheat.

lactose to α-lactose hydrate was still taking place (p. 306). Presumably the falling concentration of 'free' water in the powder and the hygroscopic nature of the residual protein combined to prevent a further rise in equilibrium relative humidity beyond this point.

The experimental results (Table 3) show that lactose commenced to crystallize in H powder only after a marked 'induction period' of the order of 1 day at 37°, 10 days at 28·5° or 100 days at 20° C. Crystallization was slightly delayed in nitrogen as compared with air, but the difference due to this cause was small at 37 and 28·5° C., the difference at 37° C. quoted in the table including the effect of a slower attainment of thermostat temperature by the powder in the large, gas-packed cans as compared with the small, air-packed tubes.

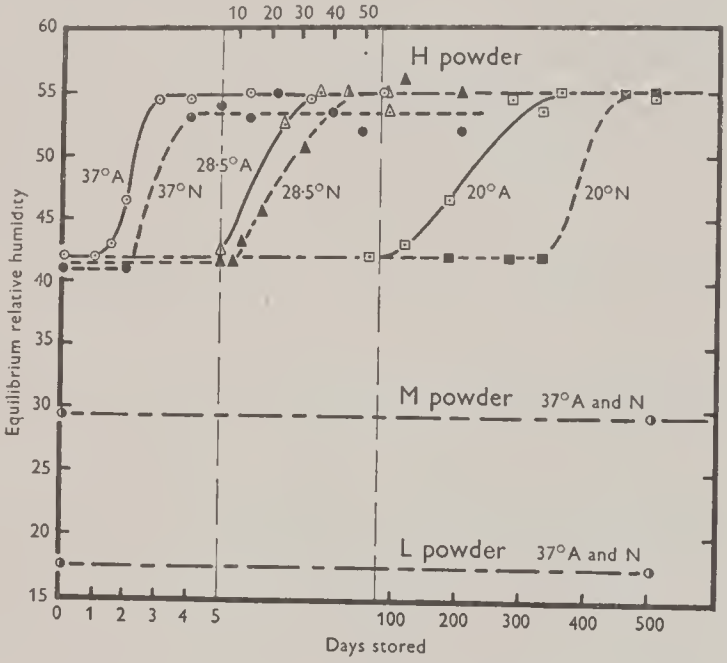

Fig. 5. The effect of the crystallization of α-lactose hydrate during storage on the equilibrium relative humidity of the powders.

Table 3. *The crystallization of lactose in separated milk powder of 'high' (7·5–7·8%) moisture content during storage*

Storage temp. (° C.)	Atmosphere	Container	Delay in onset of crystallization (days)	Increase in relative humidity complete after (days)
37	Air	Small glass tubes	1	3
37	Nitrogen	Cans	2	4
28·5	Air	Cans	5	30
28·5	Nitrogen	Cans	10	40
20	Air	Cans	100	300
20	Nitrogen	Cans	300	400

At 20° C., gas-packed H powder commenced to crystallize some 6 months later than the air-packed material, a delay which was reflected in the very appreciably slower rate of deterioration shown by this powder when judged by several different criteria (cf. Figs. 13, 16–18). Since the 'gas powder' was packed at Cambridge and the 'air powder' at the Hannah Institute, part of both packs then being stored at each laboratory, it is just possible that the air-gas difference observed might have been due to a very slight initial difference in the moisture content of the powder packed at the two laboratories, or to a small loss of moisture during the exhaust stage of the multiple gas-packing technique used (Part II, p. 296). It should be stated, however, that careful comparison of the

total moisture content of one sample of 20° C. H air-packed powder which had crystallized, with a sample of 20° C. H gas-packed powder of the same age which had not crystallized failed to show any appreciable difference in total moisture content. On the other hand, several cans of 20° H air-packed powder of the same age, which had been selected by equilibrium R.H. determination and by analysis of the headspace gas as having commenced to crystallize at different dates, showed moisture contents varying over a small range in precisely the expected manner. The balance of the evidence available therefore suggests that some cause other than slight fortuitous variation in moisture content may be responsible for the delayed crystallization of lactose in gas- as compared with air-packed H powders.

The possible influence of factors such as agitation in starting crystallization has not been explored. The data given in Fig. 5 show the condition of the particular can, or group of cans, of H powder used for the chemical investigations. Samples of powder removed from a can of which the contents had apparently just commenced to crystallize, repacked in glass tubes and held at 20° C. completed their rise in relative humidity in about 1 month.

M and L powders had not begun to crystallize even after storage for as long as 600 days at 37° C.

Conversion of β-lactose to α-lactose hydrate and decrease of total soluble lactose (J. C. D. White)

Troy & Sharp (4) observed a considerable change of β-lactose to α-lactose hydrate in milk products stored at laboratory temperature and 70% relative humidity for 5 months. Since the initial high equilibrium R.H. (*c.* 42%) of H powder gradually increased to *c.* 55% because the anhydrous α-lactose crystallized to the hydrated form, it was thought probable that, in this powder at least, β-lactose would be converted to α-hydrate, especially at the higher storage temperatures.

A polarimetric method of estimating the proportions of α- and β-lactose in milk products has been described by Sharp & Doob (5). To gain experience of the method, it was applied to fresh liquid milk and the proportions of α- and β-lactose found (38·5% α, 61·5% β at 25° C.) were very close to the β/α equilibrium ratio of 1·58/1 (38·7% α, 61·3% β) for that temperature. Examination of the fresh H, M and L powders showed that 41–43% of the lactose was in the α form. As Troy & Sharp (4) have pointed out, this higher percentage of α-lactose is to be expected since the milk would be dried at a temperature above 25° C., so decreasing the β/α ratio.

Air- and nitrogen-packed samples of each powder were examined after different periods of storage. Sharp & Doob (5) advocate the use of 'norrit' as a decolorizing-agent when examining brown, deteriorated samples, especially of dried whey, but this was not found necessary with the milk powders. The results (Fig. 6) show that in air- and gas-packed M and L powders, the proportions of α- and β-lactose were unchanged even after more than 600 days' storage at 37° C. In H powder, however, β-lactose was converted to α-lactose, presumably the hydrated form, fairly rapidly at 37° C. and more slowly at 28·5 and 20° C. until after 400 days at 37° C., 98·5% of the lactose was in the α-form.

The H samples were examined after they had been taken from the incubators and stored in bottles at 0–4° C. for some time, so that any differences in the rate of conversion of β- to α-lactose due to gas-packing may have been minimized. Nevertheless, as in the

crystallization of the anhydrous α-lactose, the absence of oxygen slowed the change, although this effect was probably due simply to the slower rise in the equilibrium R.H. of the gas-packed powders.

Sharp & Doob's method(5) also measures total lactose but they have shown that the protein precipitant used, an alcoholic solution of mercuric chloride, causes a slightly low value for the apparent percentage of total lactose. However, it was obvious that during storage of H powder there was a progressive decrease in the amount of total soluble lactose. After 400 days at 37° C., the percentage of total lactose, as estimated by this method, had decreased from 50·3 to *c.* 44 %. With three other protein precipitants, the total soluble lactose averaged 45·3 %. The reason for this decrease is discussed fully in a later section (p. 322).

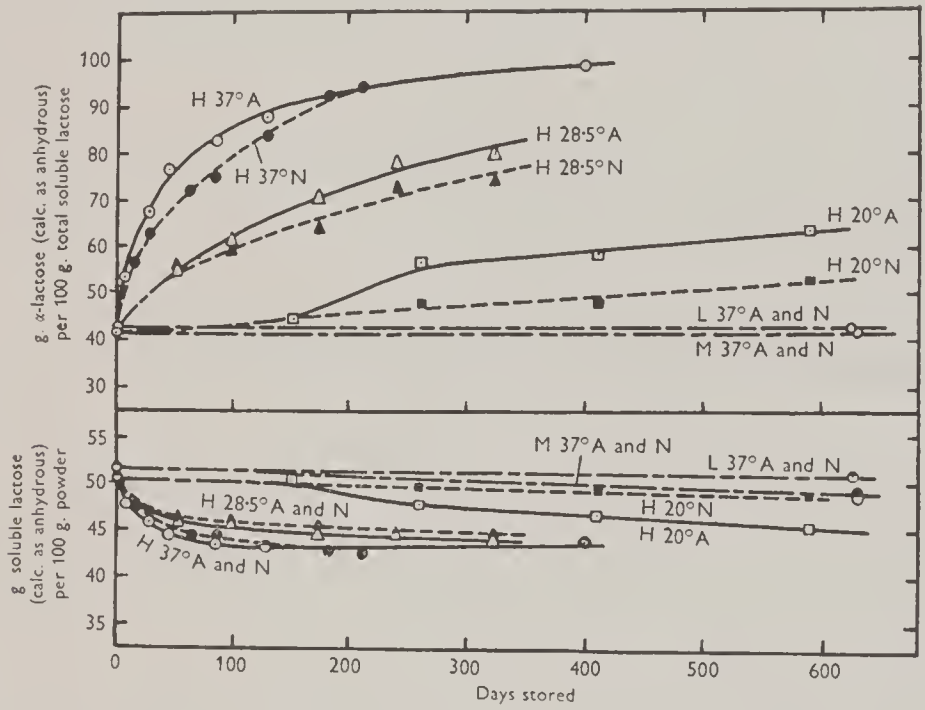

Fig. 6. The conversion of β-lactose to α-lactose hydrate, and decrease in total soluble lactose during storage.

ABSORPTION OF OXYGEN AND PRODUCTION OF CARBON DIOXIDE

As previously stated (Part II, p. 296), the powders were packed in gas-tight cans of 315 ml. capacity at the rate of 150 g. per can which, under the conditions of sealing, corresponded to a free oxygen content in the air- and gas-packed cans of 39·2 and 0·3 mg./100 g. powder respectively. For the purpose of this calculation the density of air-free separated milk powder was assumed to be 1·465(6). Prior to opening for examination a sample of the head-space gas was withdrawn from each can of stored powder by the technique previously described(3), and submitted to analysis for oxygen and carbon dioxide in a Haldane apparatus. The results, converted to absolute units by a calculation based on the constancy of the nitrogen content of the can, are summarized in Figs. 7 and 8.

Absorption of oxygen and production of carbon dioxide by H powder commenced with a marked 'induction period' of the order of 2, 20 and 200 days at 37, 28·5 and 20° C. respectively, which probably corresponded to the periods required for the crystallization of lactose under these conditions (cf. Table 3). Thereafter the rate of gas exchange was

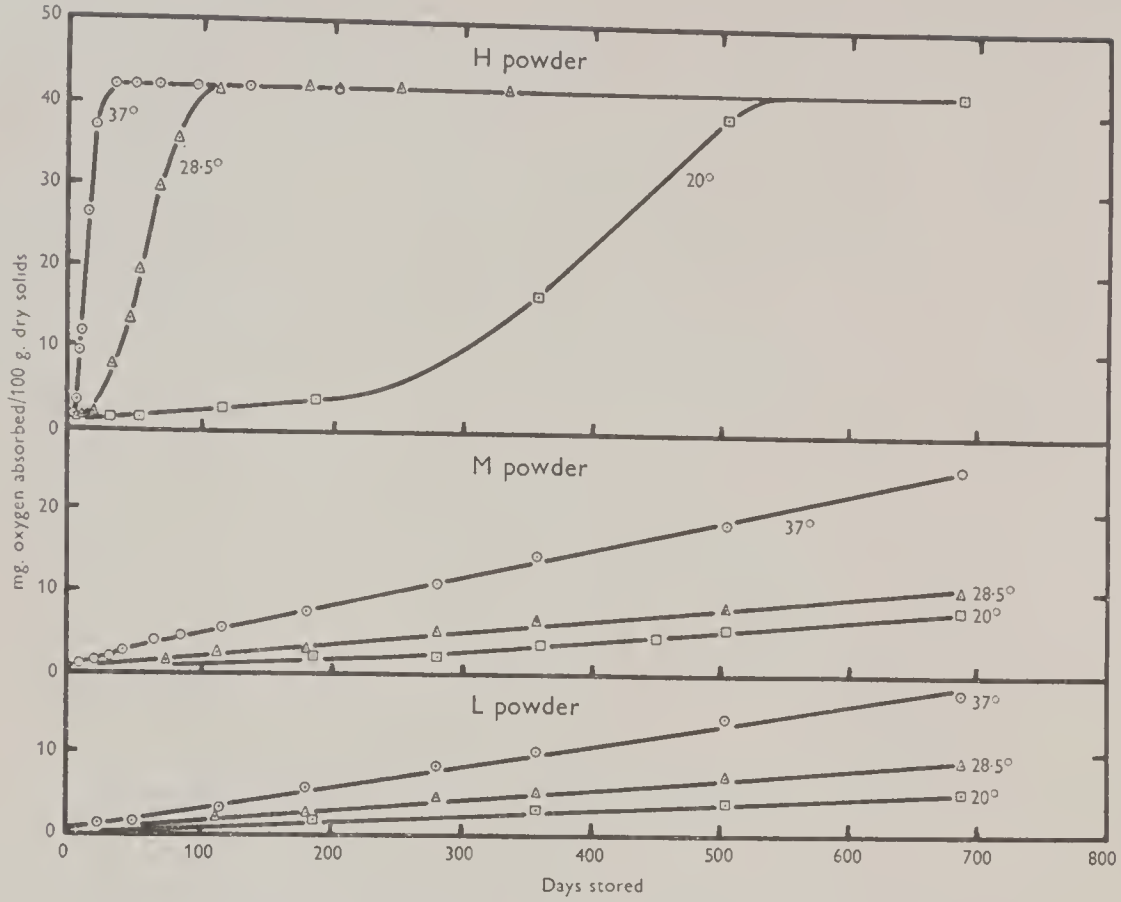

Fig. 7. Absorption of oxygen by the powders (Cambridge results).

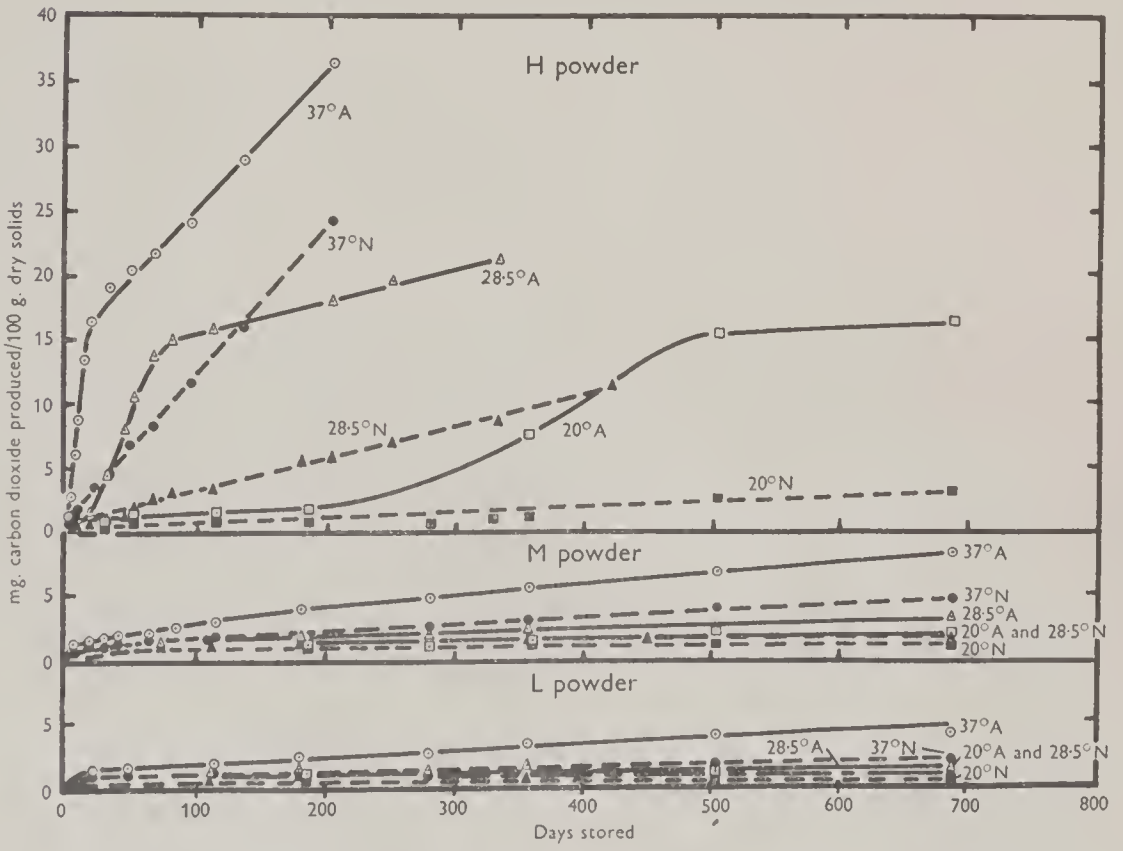

Fig. 8. Production of carbon dioxide by the powders (Cambridge results).

rapid, production of carbon dioxide being nearly ten times more rapid by air-packed than by gas-packed powders until all free oxygen in the container had been exhausted, after which the lower rate became common to both powders.

Absorption of oxygen and production of carbon dioxide by M and L powders were very much slower, and the curves were almost linear except for a small initial excess production of carbon dioxide (< 1 mg./100 g. powder) which was possibly due to equilibration of gas within and between the particles or to the decomposition of some labile minor constituent. The gradients of these lines are given in Table 4, together with approximate estimates of the corresponding values for H powder.

As with flavour, the increase in rate of gas exchange in passing from 3·0 or 5·0 to 7·6% moisture content was very great, and was greater the higher the storage temperature. The acceleration of deterioration which occurred after the commencement of crystallization of the lactose in H powder was very marked.

Table 4. *Rates of absorption of oxygen and production of carbon dioxide by the powders*

	Absorption of oxygen or production of carbon dioxide (mg./100 g. milk solids/100 days)								
	H powder			M powder			L powder		
	37° C.	28·5° C.	20° C.	37° C.	28·5° C.	20° C.	37° C.	28·5° C.	20° C.
Cambridge results									
Oxygen absorption in air-pack:									
Main reaction	250	55	15	3·5	1·5	0·9	2·5	1·2	0·7
Carbon dioxide production in air-pack:									
Main reaction	100	24	6	1·0	0·3	0·2	0·4	0·1	<0·1
After disappearance of free oxygen	11	2·5	0·5	—	—	—	—	—	—
Carbon dioxide production in gas-pack:									
Main reaction	11	2·5	0·5	0·5	0·2	<0·1	0·2	<0·1	<0·1
Hannah Institute results									
Oxygen absorption in air-pack:									
Main reaction	240	45	10	3·0	1·5	1·0	2·5	1·3	0·7
Carbon dioxide production in air-pack:									
Main reaction	96	18	6	0·7	0·3	0·2	0·4	0·1	0·1
After disappearance of free oxygen	9	2·0	?	—	—	—	—	—	—
Carbon dioxide production in gas-pack:									
Main reaction	9	2·6	?	0·4	0·2	<0·1	0·2	<0·1	<0·1

Supplementary experiment

The supply of free oxygen available in the air-packed cans of H powder had become nearly exhausted after 20 days, and completely exhausted after about 30 days, at 37° C. Subsequent storage was therefore under conditions which approximated to those in the gas-packed containers.

Since in commercial practice containers used for air-packing milk powder are not likely to be completely gas tight a small supplementary experiment providing a larger supply of oxygen (141 mg./100 g. of powder) was undertaken, using H powder only. After 60 days at 37° C. the concentration of oxygen in the cans had fallen from 20·9 to 2·5%, corresponding to the absorption of 128 mg. of oxygen per 100 g. of powder. (Part of this 'extra air' material was used in one of the rat-feeding tests described in Part IV (Exp. 8).)

Changes in solubility

Estimation of soluble solids

The solubility of the stored powders in cold (20 C.) and hot (50 or 60° C.) water was estimated at intervals at both laboratories by modifications of the method of Howat, Smith, Waite & Wright(7). In this method the powder is reconstituted with distilled water at a powder to water ratio of 1 : 9, the insoluble material is centrifuged down, and the solids content of the supernatant liquid is measured. The ratio of the dissolved solids to the total dry solids initially present, expressed as a percentage, is taken as an index of the solubility of the powder.

It had previously been observed with very soluble powders that solubility indices determined at the Hannah Institute by this method frequently exceeded the theoretical maximum of 100 by 2–3 units, an anomaly which was considered as probably due mainly to partial hydration of originally anhydrous lactose during the determination(3). It has

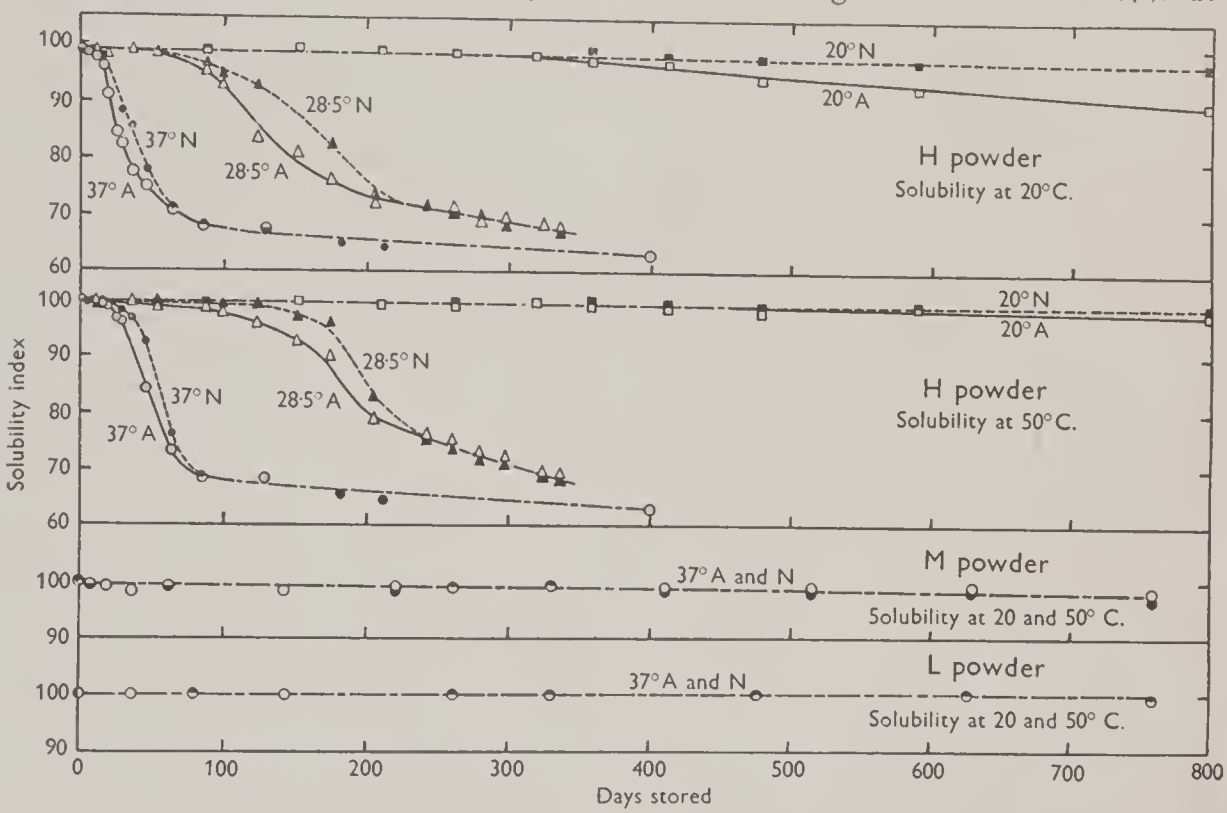

Fig. 9. Loss of solubility by the powders during storage, as determined after reconstitution in water at 20 and at 50° C. (Hannah Institute results).

therefore been usual to apply a small correction to the experimentally determined figures to allow for this error(1, 2), a procedure which has been followed at the Hannah Institute in the present work.

In the slightly different procedure adopted at Cambridge a control determination of dry weight, carried out on the reconstituted powder without centrifuging, was run side by side in each determination with that on the centrifuged sample, in order to supply the necessary correction. The deviation from 100% solubility found in the controls ranged from about 0·2 to 1·0, with a mean of about 0·5%, indicating that under the particular conditions of drying used the degree of hydration of the lactose occurring was comparatively slight.

The solubility of the fresh powders in water at 20 and at 50 or 60° C. approximated to 99 and 100% respectively. The results obtained on storage of the three powders in air

and nitrogen at 37, 28·5 and 20° C. are shown graphically in Fig. 9, while the times taken by the powders to reach various stages of insolubility are listed in Table 5. Reduction of the solubility of the powder to *c.* 65% implies complete insolubility of the protein fraction. The data show that the moisture content of the powders or, more correctly, the activity of water in the powders, exercised a decisive influence on the development of insolubility, H powder showing a greater loss of solubility after storage for 1 week at 37° C. than M or L powder after 2 years at the same temperature.

The temperature coefficient for this form of deterioration was again high, an average factor of about 5 being observed for the temperature interval 37–28·5° C., with signs of an even greater factor between 28·5 and 20° C. (Table 5). Delayed crystallization of the lactose with its accompanying increase in equilibrium R.H. (cf. Fig. 5, Table 3) probably contributed to the induction period, which was of the order of 5–10 days at 37°, 40–80 days at 28·5° and 400 days or longer at 20° C., and which preceded any loss of solubility in the

Table 5. *Loss of solubility of H powder during storage**

(Initial values 99% at 20° C.; 100% at 50 or 60° C.)

Storage temp. (° C.)	Pack	Time taken to deteriorate to the stated percentage solubility (days): In water at 20° C. 95%	90%	80%	70%	In water at 50 or 60° C.† 95%	90%	80%	70%
		Hannah Institute results							
37	Air	16	20	28	70	28	36	52	74
	Nitrogen	20	28	41	70	39	48	58	78
28·5	Air	86	105	150	260	130	165	200	310
	Nitrogen	100	135	180	260	170	190	220	310
20	Air	500	—	—	—	—	—	—	—
	Nitrogen	—	—	—	—	—	—	—	—
		Cambridge results							
37	Air	9	12	18	55	22	28	38	64
	Nitrogen	11	14	22	55	25	32	41	65
28·5	Air	52	68	110	240	100	140	210	320
	Nitrogen	65	82	130	260	120	160	230	340
20	Air	450	610	—	—	—	—	—	—
	Nitrogen	620	—	—	—	—	—	—	—

* Absence of a figure in the table signifies a period of over 700 days.

† Solubility in hot water was measured at 50° C. at the Hannah Institute, and at 60° C. at Cambridge.

H powder. On the other hand, later experiments with artificial dialysed protein-sugar systems stored at a constant relative humidity also showed a marked induction period (8), which indicates that the crystallization of lactose cannot have been the main factor in delaying the onset of loss of solubility of the milk powders. This point is treated more fully in a subsequent section (p. 332).

Insolubility in water at 50 or 60° C. developed much later than insolubility in water at 20° C., and can be considered as representing a more advanced stage of deterioration (Fig. 9, Table 5). Partly deteriorated samples which, although of poor solubility in cold water, still remained quite soluble in hot, thus displayed an interesting similarity to roller-dried powder in which some protein insolubility has been caused by severe heat treatment during drying. Wright (9) has shown that the difference in solubility of roller-dried powders when estimated at 20 and 50° C. is a measure of the amount of protein rendered insoluble by dry heat. Whether or not the mechanism of deterioration by excessive heat during and immediately after removal of most of the water in the roller-

drying process is the same as that produced by storage of powder at too high a moistur content and storage temperature is not yet known, and requires investigation.

The gas-stored samples developed insolubility definitely more slowly than the air stored samples but the differences were never very great and can be accounted for, i part, at least, by the slightly delayed crystallization of lactose in the gas-stored powders

Table 5 discloses a considerable difference in the rates at which powders stored at th two laboratories, ostensibly under the same conditions, developed insolubility. The effec seems too large to be accounted for entirely by slight differences in effective storag temperatures, and is probably due in part to the different mechanical means employed a the two laboratories to reconstitute the powders. It is well known that the degree o stirring or shaking to which a milk powder is subjected during reconstitution markedl influences its apparent solubility (7).

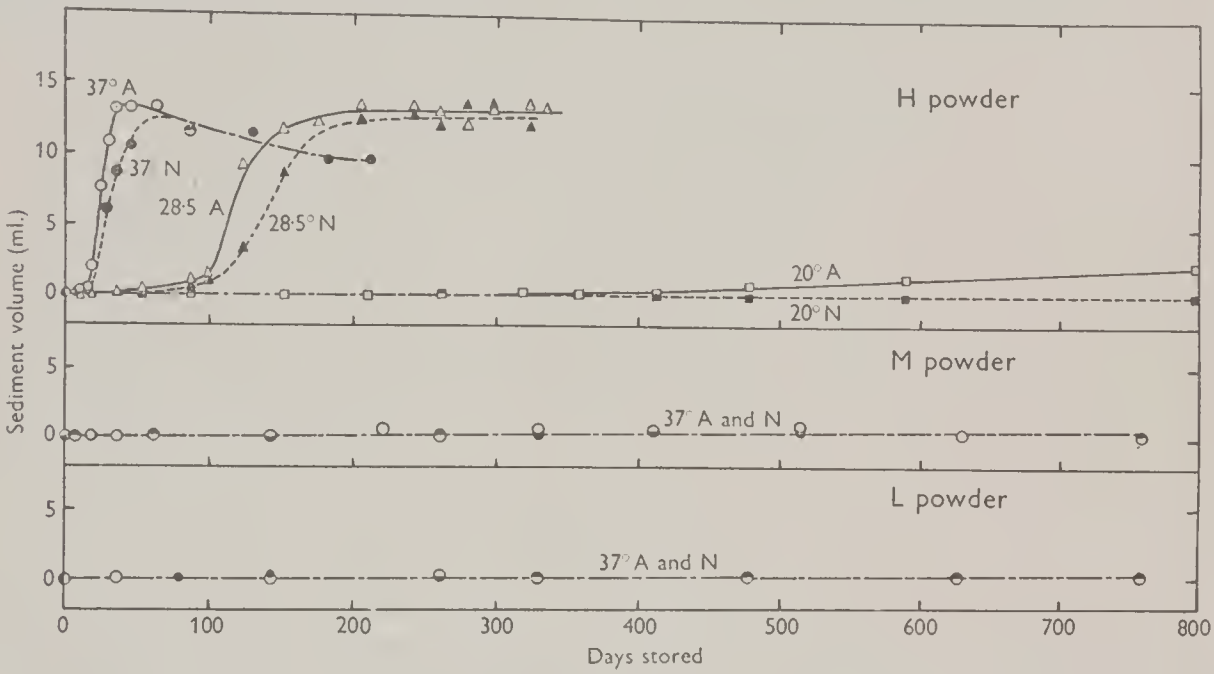

Fig. 10. Loss of solubility by the powders as measured by increase in sediment volume after reconstitution in water at 20° C. (Hannah Institute results).

Measurement of sediment volume

The American Dry Milk Institute Inc. has developed a standard 'sediment' solubility method, but as the most recent description of this method (10) specifies apparatus which was unobtainable at the time, the procedure was altered slightly. The modified method as used at the Hannah Institute, was as follows: 20 g. powder were added to 200 ml distilled water at 20° C. contained in a 500 ml. wide-mouthed bottle. (Before adding the powder, the bottle was shaken to wet the sides and prevent the powder sticking to the bottle.) The bottle was shaken for 30 sec., allowed to stand for 5 min., shaken again for 30 sec. and a centrifuge tube of 50 ml. capacity filled to the mark with the 'milk'. The tube was then centrifuged for 15 min. at 1000 r.p.m. (216 *g*). The supernatant liquid was removed by suction, 25 ml. water at 20° C. added and the tube shaken for 30 sec., taking care to dislodge all the sediment. The tube was filled to the mark with water at 20° C and finally centrifuged for 15 min. as before. The volume of the sediment thus obtained gave a measure of the solubility. The results obtained by the application of this method to the stored powders are shown in Fig. 10.

These estimations bore out the conclusions obtained by the more absolute method. It was found that the volume of sediment obtained from the H powder at 37° C. decreased after the powder had deteriorated to its maximum insolubility (i.e. as judged by sediment volume). This decrease was probably due to some physical change in the coagulated protein particles which enabled them to 'pack' more closely when centrifuged. It was also noticed with severely deteriorated H powder, that some insoluble material was present on the surface of the supernatant liquid after centrifuging. When this layer was broken up and the suspension recentrifuged, the amount of floating material was reduced but could not be removed completely. The trace of fat present may have carried up insoluble protein to the surface and this was probably a minor contributing cause of the decrease in sediment volume.

CHANGES IN THE DISTRIBUTION OF THE SOLUBLE NITROGEN (J. C. D. WHITE)

It has been shown that the solubility of L and M powders remained almost unaltered even after storage for 700 days at 37° C. H powder, however, although showing little change in solubility when stored at 20° C., rapidly became insoluble when stored at 28·5 and 37° C. whether packed in air or nitrogen. As the loss of solubility of a milk powder is largely due to a decrease in the solubility of the milk proteins, it was decided to determine the distribution of the *soluble* nitrogen and thus indirectly find which fractions of the protein were becoming insoluble and also if soluble nitrogenous decomposition products were being formed. Such an examination would indicate whether the deterioration involved only the casein or whether lactalbumin and lactoglobulin were also involved.

Examination of the fresh powders

For the determination of the initial nitrogen partitions, the fresh powders were reconstituted with water at 20° C. in the manner prescribed for solubility estimation (7) and the 'milk' made up to a known volume. The various protein fractions were separated and estimated by the methods developed by Rowland (11, 12) for liquid milk except that a micro-Kjeldahl procedure (13, 14) was used where possible. The following direct estimations were made: (1) total nitrogen, (2) non-casein nitrogen, (3) non-protein nitrogen, and (4) proteose-peptone plus non-protein nitrogen. From the results so obtained, the following were calculated: casein nitrogen (1)–(2), lactalbumin plus lactoglobulin nitrogen (2)–(4), and proteose-peptone nitrogen (4)–(3).

The proteose-peptone plus non-protein nitrogen was measured by heating the 'milk' for 15 min. at 95° C. to denature the lactalbumin and lactoglobulin. The pH of the 'milk' was then adjusted to 4·6–4·7 (as in (2) for the precipitation of casein alone) to precipitate the denatured 'heat-coagulable' proteins with the casein. The filtrate thus contained only proteose-peptones and non-protein nitrogen. Direct estimations of lactoglobulin were also made.

The nitrogen partitions for the H, M and L fresh powders are given in Table 6. Rowland's (15) average values for the nitrogen partition of fresh milk and Ashworth & Van Orden's (16) data for spray-dried skim milk are included in the table. Comparison of the average values for the three powders with those for fresh milk shows that the percentage of the total nitrogen in the form of lactalbumin plus lactoglobulin in the powders was only 5·5 as compared with 12·5 for fresh milk. This difference was compensated by an apparent increase of 3·7% in casein nitrogen and increases of 2·3 and 0·9% in proteose-peptone

and non-protein nitrogen respectively, i.e. a combined increase of 6·9%. Ashworth & Van Orden's data for other spray-dried milks show a similar but more severe deviation from the nitrogen partition of fresh milk in that almost the whole of the lactalbumin plus lactoglobulin appeared to be denatured, thus causing a corresponding apparent increase in the casein fraction.

Since the milk was dried by the Gray-Jensen spray process, the milk or powder was not subjected to high temperatures during the actual dehydration (17, 18, 19). The deviation of the nitrogen distributions of the fresh powders from that of normal liquid milk would thus depend largely on the temperature [165° F. (74° C.)] and duration (*c.* 30 min.) of the pre-heat treatment, described in detail in Part II.

Rowland (20, 21) has examined the effect of various 'temperature-time' treatments on the nitrogen distribution of milk. He states that lactalbumin and lactoglobulin are rapidly denatured at temperatures of 75° C. and above, and that there is no change in non-protein nitrogen content on heating at temperatures up to 100° C. On continued

Table 6. *Distribution of nitrogen in the fresh powders used in the present work as compared with that found by others for liquid milk and spray-dried skim milk*

	mg. N per g. milk solids					Percentages of total nitrogen					
	Powder				Av. of 32 samples of spray-dried skim milk*	Powder				Av. of 32 samples of spray-dried skim milk*	Liquid milk†
Constituent	H	M	L	Av.		H	M	L	Av.		
Total N	54·1	53·8	55·2	54·5	58·13	100·0	100·0	100·0	100·0	100·0	100·0
Casein N	44·1	44·1	46·0	44·7	52·80	81·5	81·9	83·3	82·2	90·75	78·5
Non-casein N	10·0	9·7	9·2	9·6	5·50	18·5	18·1	16·7	17·8	9·25	21·5
Lactalbumin + lactoglobulin N	3·4	3·3	2·3	3·0	0·23	6·2	6·2	4·1	5·5	0·40	12·5
Proteose-peptone N	3·4	3·1	3·9	3·5	2·30	6·2	5·8	7·0	6·3	3·95	4·0
Non-protein N	3·3	3·3	3·1	3·2	3·06	6·1	6·1	5·6	5·9	5·26	5·0

* Ashworth & Van Orden's (16) mean values for thirty-two samples of spray-dried skim milk.

† Rowland's (15) average values for liquid milk.

heating (30 min.) at 95 and 100° C., very small amounts of proteose-peptone substances are produced. Menefee, Overman & Tracy (22) found that in the preparation of evaporated and condensed milk, pasteurization at 145° F. (62·8° C.) for 30 min. produced no significant differences in nitrogen distribution. On the other hand, fore-warming to 150° F. (65·6° C.) caused slight 'coagulation' of lactalbumin, whereas pasteurization at 190° F. (87·8° C.) for 30 min. and fore-warming to 203° F. (95° C.) both caused complete 'coagulation' of lactalbumin and probably of some lactoglobulin.

These investigations support the view that the changes in the nitrogen distribution of the fresh powders were largely caused by the pre-heat treatment of the liquid milk.

Examination of the stored powders

Using the analytical procedures mentioned above, the nitrogen distribution of H powder was examined at intervals during storage at 37 and 28·5° C. In these analyses, the reconstituted milk was centrifuged to remove the insoluble protein. From the known moisture content of the powder and the percentage solubility of the milk solids, it was possible to calculate the weight of supernatant liquid after centrifuging and thus express

the results as mg. of soluble nitrogen per g. of dry milk solids. Figs. 11 and 12 illustrate the changes in the distribution of the soluble nitrogen during storage of H powder at 37 and 28·5° C. These changes may be summarized as follows:

(1) The same changes took place at both storage temperatures although at a much slower rate at 28·5° C. As would be expected, they ran concurrently with the decrease in solubility of the powder.

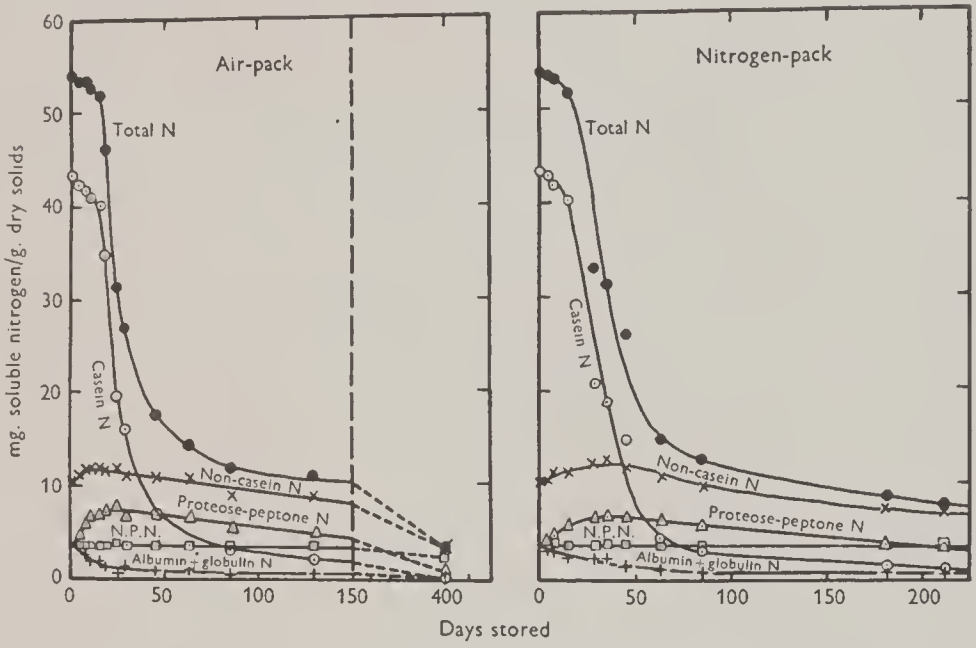

Fig. 11. Changes in the distribution of the soluble nitrogen of H powder stored at 37° C.

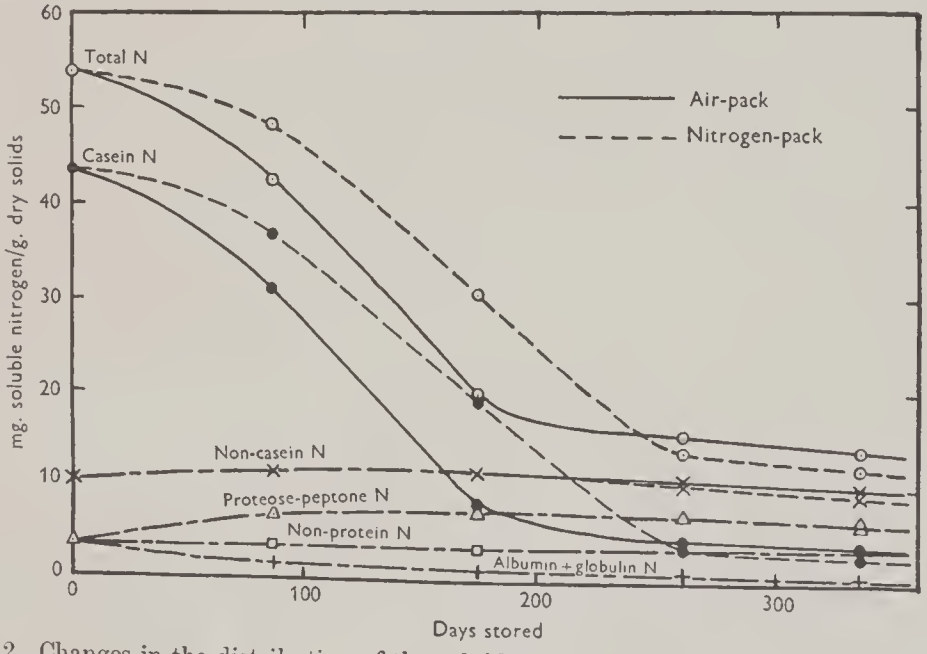

Fig. 12. Changes in the distribution of the soluble nitrogen of H powder stored at 28·5° C.

(2) As storage progressed, the casein rapidly became insoluble and was completely insoluble after 400 days at 37° C. Since the casein nitrogen constituted about 81 % of the total nitrogen, the decrease in soluble casein was closely paralleled by a decrease in total soluble nitrogen.

(3) The proteose-peptone fraction increased to about twice its initial value after about 24 days at 37° C. and also after about 90 days at 28·5° C. and then decreased slowly until

after 400 days at 37° C. only about one-quarter of the initial amount remained i solution.

(4) The level of non-protein nitrogen remained constant showing that there was n severe protein decomposition.

(5) The lactalbumin plus lactoglobulin slowly decreased in solubility and eventuall became entirely insoluble (see data for H powder stored for 400 days at 37° C., Fig. 11)

(6) There was a small initial increase in non-casein nitrogen. Thereafter the amoun of non-casein nitrogen decreased slowly.

The interpretation of these results is complicated by the fact that the method o nitrogen fractionation, which was developed in the first place for liquid milk, became less efficient when applied to a deteriorated powder. The lactalbumin plus lactoglobulin nitrogen results as determined for H air-packed powder would indicate that these combined fractions decreased from an initial value of 3·3 to 0·0 mg. soluble nitrogen/g dry milk solids after 400 days' storage at 37° C. These figures were obtained by subtracting the proteose-peptone plus non-protein nitrogen from the non-casein values (cf. p. 313). However, direct estimation of the soluble lactoglobulin nitrogen showed that this fraction remained fairly constant at a value of about 2 mg. nitrogen/g. dry milk solids up to 129 days' storage at 37° C. and then decreased to 0·7 mg./g. solids after 400 days storage. Ashworth & Van Orden(16) and Menefee *et al.*(22) are of the opinion that the method of estimating lactoglobulin (saturation of a solution containing proteose-peptones, lactalbumin, lactoglobulin and non-protein nitrogen with magnesium sulphate) is not accurate enough, since they found small variable amounts of lactoglobulin in samples of heated milk and milk powders which apparently contained no heat-coagulable protein. Despite this possible inaccuracy, the *estimated* lactoglobulin nitrogen remained at a fairly constant level simultaneously with a redistribution of the other nitrogen fractions and it seems reasonable to conclude that the same uncontaminated lactoglobulin fraction was being estimated each time. If this supposition is correct, then the *calculated* lactalbumin plus lactoglobulin results are too low, since, after 10 days' storage, the lactalbumin values for H air-packed powder at 37° C., obtained by difference, become negative. If, then, the lactoglobulin analyses are assumed correct, the most likely error in the fractionation is that the values for the proteose-peptone plus non-protein nitrogen are too high. A possible explanation may be that lactalbumin and lactoglobulin lose their property of being coagulated by heat and these fractions would then be included with the proteose-peptone and non-protein nitrogen (cf. p. 313). The anomaly of 'negative' lactalbumin values was found also in the nitrogen-packed series and the more deteriorated the powder, the greater was the discrepancy in the analytical results. A similar but even greater discrepancy was found when the fractionation procedure was applied to evaporated milks which had been stored for 10–11 years. Further work is being carried out to find why the separation fails with 'deteriorated' milk protein.

Because of these discrepancies, it is impossible to state quantitatively the changes in solubility of the lactalbumin and lactoglobulin fractions. However, it is probable that most of the lactalbumin of H powder was rendered insoluble after 10 days' storage at 37° C. for the air-pack, and after 28 days for the nitrogen-pack. The direct lactoglobulin estimations suggest that this protein remained soluble until extreme deterioration of the powder was reached after 400 days' storage at 37° C.

The small initial increase in non-casein nitrogen seems real since it was detected in air-

and nitrogen-packed powders both at 28·5 and 37° C. Consequently, at least part of the increase of the proteose-peptone fraction must have been due to casein decomposition. The analytical data do not indicate whether the lactalbumin and lactoglobulin fractions were also involved in the formation of proteose-peptones.

Lampitt & Bushill(23) have also investigated the distribution of the different protein constituents between the soluble and insoluble fractions of milk powders of high moisture contents, stored at 30° C. They state that, contrary to the findings of Supplee & Bellis(24), a proportion of the casein remained soluble after storage, although in one instance the casein decreased from 83·1 % of the total soluble protein to only 2·4 %. It has been shown in the present work that the casein of a milk powder with a high moisture content can become completely insoluble after prolonged storage at a high temperature. Moreover, Lampitt & Bushill(23) say that a relatively large proportion of the lactalbumin and lactoglobulin fractions remained soluble. However, the technique of their analyses did not differentiate between proteose-peptones and the 'heat-coagulable' proteins, and thus soluble proteose-peptones would be estimated as lactalbumin plus lactoglobulin.

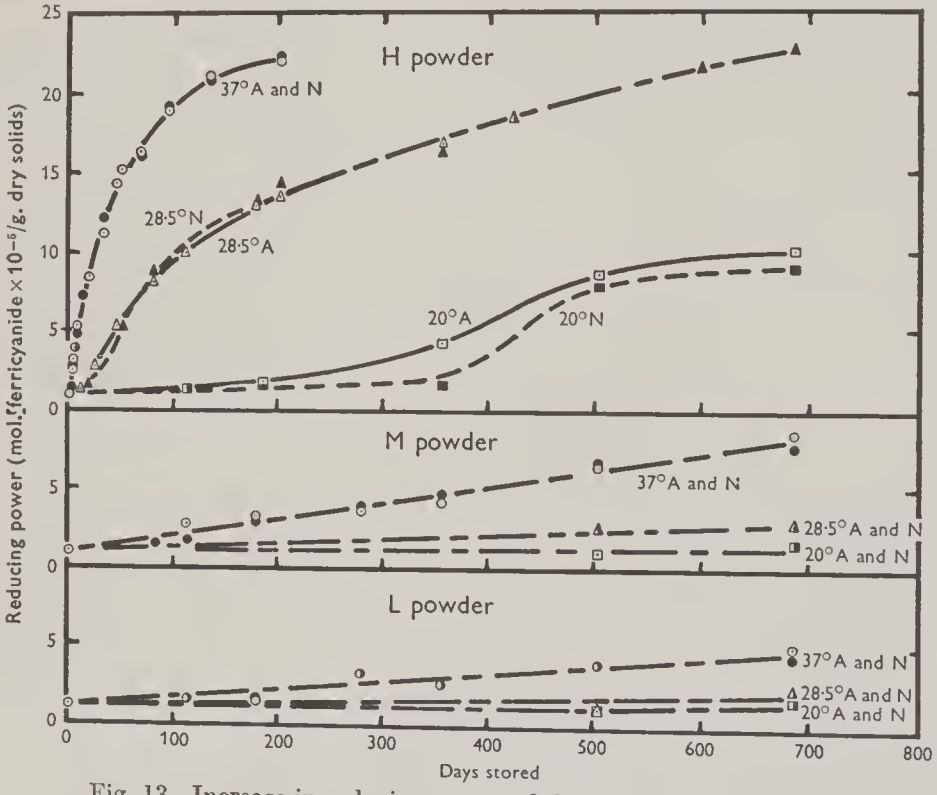

Fig. 13. Increase in reducing power of the powders on storage.

REDUCING POWER OF THE MILK TOWARDS POTASSIUM FERRICYANIDE (C. H. LEA)

Determinations of the reducing power of the fresh and stored milk powders were carried out according to a modification of the method of Chapman & McFarlane(25), which is discussed more fully elsewhere(26). This method depends on the reduction of potassium ferricyanide solution by heating with the powder at 70° C. and pH 5·0, after which the protein is precipitated by trichloroacetic acid and the ferrocyanide produced estimated colorimetrically after development with ferric chloride. Since the reaction now appears to be due to the formation and degradation of a protein-sugar complex, rather than to a simple denaturation of protein with consequent exposure of reducing groups as originally believed, the values obtained directly are expressed as mol. $\times 10^{-5}$ ferricyanide

reduced per g. dry milk solids, rather than in terms of cysteine or glutathione as in t original method.

The results, which are summarized in Fig. 13, stress once more the deleterious effect a high moisture content in the powder particularly when combined with a high stora temperature. An induction period of the order of 2, 20 and 300 days, with the powder highest moisture content stored at 37, 28·5 and 20° C. respectively is probably attr butable, in part at least, to delayed crystallization of the lactose (cf. Table 3), as probab also is the comparatively slight difference between powders stored in air and nitrog at the two latter temperatures. No appreciable air-nitrogen difference was detectable 37° C. The reducing power of M and L powders increased but slowly, and in a linea manner, on storage, no appreciable difference being detectable between the air- a nitrogen-packs.

The effects of moisture content and temperature of storage on the development reducing power in separated milk powder are thus, on the whole, rather similar to t effects of these factors on a number of the other physical and chemical criteria studie and have not been tabulated separately.

It will be shown later (p. 334) that the ferricyanide-reducing power of the powde which can easily be determined as a routine procedure, appears to be closely related to t amino-sugar reaction, which itself seems to be a major factor in the causation of no fatty deterioration in a variety of foods (27).

Changes in pH, in base-binding capacity and in formol titration (C. H. Lea)

The discoloration of evaporated milk during sterilization has been considered by som workers to be due, in part at least, to interaction between lactose and protein, the ald hyde group of the sugar becoming attached to the protein molecule, possibly at fr amino-groups (28, 29). Others have attributed it to a protein-catalysed caramelization the lactose (30, 31).

As a result of the blocking of free amino-groups by such a reaction the pH of the mil or milk protein might be expected to fall, and its base-binding power to increase, while th content of free amino-groups as measured by formol titration or by the Van Slyke reactio with nitrous acid might be expected to decrease. In fact, the pH of milk is known t decrease and the titratable acidity to increase when liquid milk is strongly heated (32 Recent work, however, has shown that considerable quantities of lactic and of volati acids including formic are produced, presumably from lactose, when milk is heated und sterilizing conditions (33), and an increase in the acidity of milk on heating cannot therefor be taken as evidence of a reaction involving the free amino-groups of protein. Furthe more, the caramelization of pure sugar is known to produce a fall in pH.

Kass & Palmer (34) have already attempted to use the formol titration method, with o without oxalate (stated by Pyne (35) to eliminate interference by phosphate) as an inde of protein-lactose combination in liquid milk during sterilization, but with little succes and, on the basis of their results, question the validity of the formol titration of milk a a means of seeking evidence for an aldose-casein condensation. Gould & Frantz (32), i a paper which appeared while the work here reported was in progress, observed *increas* in formol titration when milk was heated at high temperatures, but the change wa slight and did not appear to be closely related to browning of the milk. Since the additio of oxalate greatly reduced the increase in formol titration it was concluded that the sal

of the milk were responsible for part of the observed effect. In a still more recent paper Gould, Weaver & Frantz (36) find that the formol titration of evaporated milk is essentially unaffected by storage.

In the present work an attempt has been made to follow interaction between protein and sugar in the experimental dried milks during storage, from the protein side by estimation of free amino-groups by formol titration and by the Van Slyke method, and from the sugar side by estimation of the quantity of sugar combined with the protein.

Method

For investigation of changes in pH, in base-binding power and in formol titration, the stored milk powders were reconstituted with water at 20 or 60° C. to 9·2% solids content, and pH measured by means of the glass electrode. 25 g. of the milk were then brought to pH 8·5 by the stepwise addition, at a standardized rate, of 0·1 N sodium hydroxide, 10 ml. of 25% formaldehyde (previously adjusted to pH 8·5) were added and titration was continued back to, and slightly beyond, pH 8·5. The amount of formaldehyde used was chosen to conform to the recommendations of Levy (37). Corrections for the change in ionic strength resulting from the addition of alkali to the milk and from dilution with the formaldehyde solution were found to be negligible. From the smooth curve obtained, the base-binding capacity of the milk (as milli-equivalents NaOH bound/g. of separated milk solids), and the formol titration (as milli-equivalents NaOH/g. of separated milk solids required to bring the pH back again to 8·5 after the addition of formaldehyde) were calculated. Changes in these values, and in the pH of the reconstituted milk, resulting from the storage of H powder at 37° C. in air or nitrogen for periods up to 203 days are given in Fig. 14.

While titration to an end-point at pH 8·5 is frequently recommended for proteins (38), and a number of proteins certainly appear to give maximum values for the formol titration in this region, e.g. β-lactoglobulin (39), in the case of milk and dialysed milk the displacement of the titration curve produced by formaldehyde seems to be appreciably greater at pH 9·0 than at pH 8·5, as found by Kekwick & Cannan (40) for egg albumin, and several values have therefore been determined also by titrating to this pH. Titration was not continued beyond pH 9·0 and it is not certain that an absolute maximum had been reached. Melnick, Oser & Weiss (41) have recently preferred to terminate the formol titration of enzymic hydrolysates of proteins at pH 9·5.

Kekwick & Cannan (40) have provisionally identified the groups combining with formaldehyde in the formol titration of egg albumin with the ε-amino-groups of the lysine residues, and believe that α-amino- and other groups should not interfere to any great extent (cf. also Cannan (38)). When the formol titrations obtained on the fresh, unstored control powders (M and H), in the present experiments are calculated to the lysine content of the dry, separated milk solids, values of 2·69% lysine when measured at pH 8·5, or 2·75% when measured at pH 9·0 are obtained. The amount of lysine found by direct microbiological assay on M control powder was 2·65% (Part V) and 2·9% has been reported in the literature for separated milk powder (42).

A number of the samples of stored powder, after reconstitution at 20 or 60° C., were centrifuged for 15 min. at 3000 r.p.m. and the supernatant liquor separated and titrated as above, to give the 'soluble' formol titration. Data for these samples (Fig. 14) reflected mainly the loss of solubility of the protein on storage, as already shown by

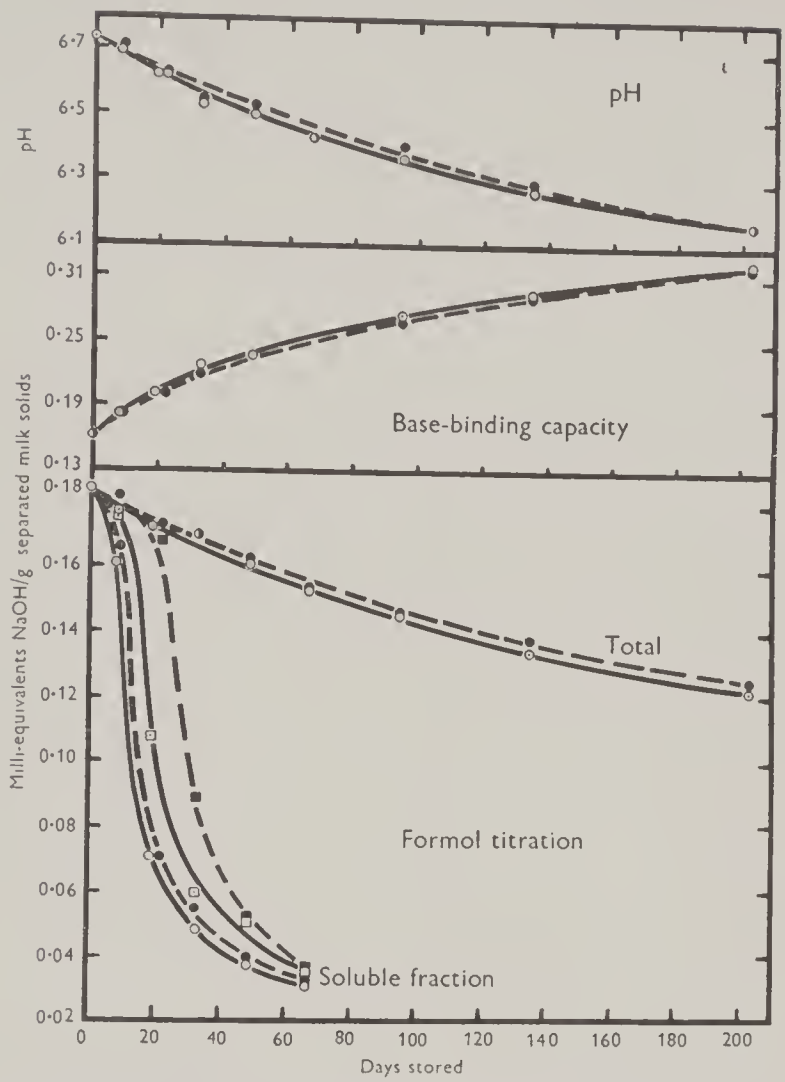

Fig. 14. Changes in pH, in base-binding capacity and in formol titration of H powder on storage at 37° C. Full line = air-stored, broken line = gas-stored samples.

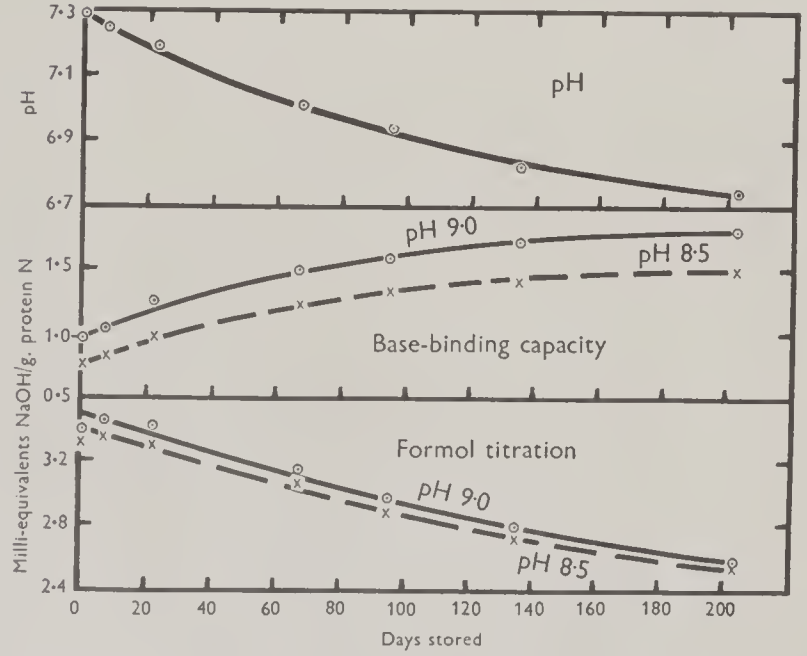

Fig. 15, Changes in pH, in base-binding capacity and in formol titration of dialysed protein from gas-stored H powder. Titrations ended alternatively at pH 8·5 or 9·0.

direct determination (Fig. 9), but served also to show that a very large proportion of the formol titration of the milk was, in fact, due to coagulable protein, or to salts carried down with it.

The progressive fall in formol titration of the high moisture powder on storage at 37° C. was quite definite (Fig. 14), but the change was rather slow, amounting only to about one-third of the initial value after storage for 203 days. Furthermore, the formol titration showed little correlation with loss of solubility, which followed a characteristic S-shaped curve (Fig. 9), or with ferricyanide-reducing power (Fig. 13).

Dialysed milk

An attempt was made to remove anomalies by simplifying the system under investigation, by dialysing several samples of reconstituted, gas-stored, high moisture powders in cellophane at 0° C. prior to potentiometric titration. The materials titrated in this case retained the whole of their protein but only about 37–42% of the original weight of the dry, separated milk solids. A correction was made for the small change in pH produced by the change in ionic strength during the determination, by carrying out a blank titration with KCl in place of NaOH. It is obvious from Fig. 15 that removal of dialysable constituents did not greatly affect the general result. The formol titration of the dialysed control milk as carried out at pH 8·5 and 9·0 could be calculated to a lysine content of the milk protein (assumed to contain 15·7% nitrogen) of 7·8 or 8·0%, or of the dry separated milk solids of 2·6 or 2·7%, values which are again of the expected order of magnitude. However, the fall in formol titration on storage was again almost linear and corresponded to a reduction of only about one-quarter of the initial value after storage at 37° C. for 203 days. The observed increase in base-binding capacity could also be accounted for by the loss of the free amino-groups of 20–25% of the initial content of lysine. The increase in base-binding capacity on storage, when measured after dialysis, was, however, only about one-quarter of that found for the undialysed milk, which indicates that very appreciable quantities of dialysable acids, possibly lactic and formic as produced in heated liquid milk(33), are formed during storage of the high moisture powder at 37° C.

Conclusions

The formol titration of the fresh, unstored separated milk powder whether measured before or after dialysis, was in approximate agreement with the value expected on the assumption that the formol titration is due solely to free amino-groups of the protein, consisting mainly of the ϵ-amino-groups of the lysine residues.

On storage of separated milk powder of high moisture content at 37° C. for nearly 7 months, a progressive fall in pH, an increase in base-binding capacity and a decrease in the formol titration of the reconstituted material were observed.

The reduction in formol titration during storage, as measured on the undialysed and dialysed milk, was of the order of one-third and one-quarter respectively of the initial value. The increase in base-binding capacity of the dialysed material was also of the order expected, on the assumption that free amino-groups of the protein were being inactivated. The increase in base-binding power of the undialysed milk, however, was considerably larger, indicating the probable production of appreciable quantities of dialysable acid during storage.

The formol titration method, therefore, provides definite evidence of the occurrence

during the storage of milk powders of high moisture content of a reaction involving the free amino-groups of the protein. As will be shown in a later section, however, the results obtained by this method are not in good agreement quantitatively with those obtained by other and perhaps more reliable methods (cf. p. 331).

The protein-sugar reaction (C. H. Lea)

Preparation of the samples for analysis

In order to free the milk from traces of ammonium salts, urea, or other non-protein nitrogenous substances likely to interfere with the Van Slyke determination, and from the large excess of uncombined sugar and from traces of non-sugar reducing substances which would prevent direct estimation of the quantity of sugar combined with the protein, the stored milk powders (5·0 g. dry weight basis) were reconstituted in water at about 20° C., transferred to narrow cellophane sacks and dialysed at 0° C., by a standardized procedure, against frequent changes of distilled water. Under the conditions used, dialysis was virtually complete in less than 3 days, but was normally continued for 5 days, in the presence of a little toluene, to ensure the removal of all free sugar. The dialysed material, which varied from a white, milky fluid without sediment in the case of separated fresh milk or of a control powder, to a suspension of brown, insoluble particles in an almost clear fluid in the case of a badly deteriorated powder, was made up to a total volume of 100 ml., and stored at 0° C. If not used within 2 days the material was discarded and a fresh batch dialysed.

Portions of the well-mixed solution or suspension were weighed out for determination of solids content, and of nitrogen content by the macro-Kjeldahl method. Difficulty in sampling suspensions of the deteriorated products for the estimation of free amino-nitrogen and of combined sugar was overcome by the use of a rapidly emptying, stemless pipette which delivered approximately 4 g. of the well-mixed sample with a reproducibility within the limits of accuracy of the chemical determinations.

Increase in weight of the undialysable fraction

Determination of the solids content of moist materials containing protein and sugar is notoriously a matter of some difficulty as, owing to decomposition, losses recorded during normal high temperature drying procedures tend to be too high.

Two methods of drying were therefore investigated. As a rapid, routine, high temperature procedure, about 20 g. of the dialysed material were dried as rapidly as possible in a large aluminium dish (with cover) on a steam bath, followed by heating for 3 hr. in an air oven at 100° C. Reproducibility was reasonably good and the values obtained were virtually unaffected when drying was prolonged for a further 2–3 hr. In the second method the samples were freeze-dried and subsequently dried over phosphorus pentoxide at 37° C. *in vacuo* to constant weight, a period of approximately 1 week being required. Since the differences between the results obtained by the two methods were comparatively small, the simple, rapid method has been used for routine examination of the stored powders.

The experimental values which have been plotted in Fig. 16 show that, as the result of deterioration during storage, the amount of undialysable material in H powder increased by quantities ranging up to 0·8–0·9 g./g. of non-dialysable ('protein') nitrogen, corresponding to an increase of nearly 5% of the weight of the original dry, separated

milk solids. It will be shown later that this increase in weight of the undialysable fraction of the milk is nearly sufficient to account for the observed loss of Van Slyke amino-nitrogen on the assumption that one molecule of lactose has reacted with each free amino-group destroyed, and is approximately sufficient to account for the combined sugar found by direct estimation (p. 325) and for the observed decrease in total soluble lactose (p. 307).

Degradation of the initial reaction product with formation of dialysable or volatile fragments (e.g. formic and lactic acids, carbon dioxide, water, etc.) would reduce the observed gain in weight, while reaction of any monosaccharide which might be present in very small quantity together with the lactose (cf. p. 331) would also reduce the expected increase in weight.

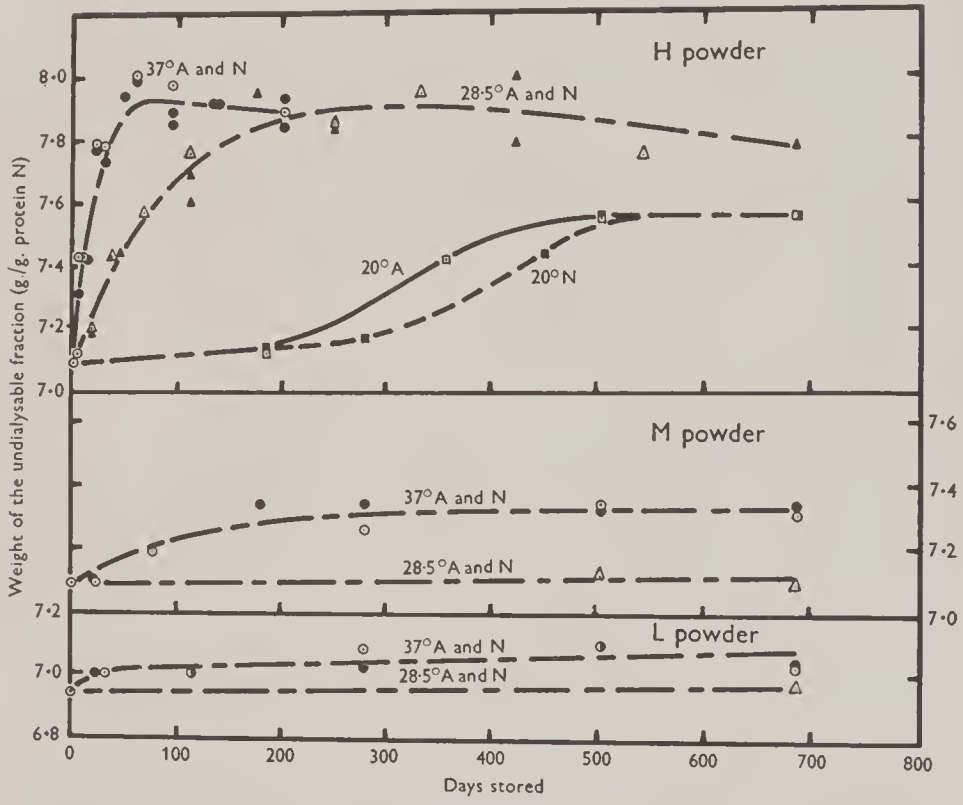

Fig. 16. Increase in weight of the undialysable fraction of the milk on storage.

Nitrogen content of the undialysable fraction

Analysis of the undialysable fraction of the milks for nitrogen content showed that no decrease in undialysable nitrogen occurred on storage, any slight tendency being rather in the direction of an increase. Long-stored, insoluble samples contained 93·5–95·5% of their total nitrogen in an undialysable form. Soluble samples showed an undialysable nitrogen content which varied erratically between 92·5 and 95%, the range of values recorded probably being due to varying permeability or to occasional microscopic leaks in the cellophane sacks used for dialysis. Calculation of all results to the undialysable ('protein') nitrogen basis largely eliminated any small errors which might have arisen from this cause, and also facilitated rejection of the occasional grossly faulty sack. The observed contents of undialysable nitrogen are in agreement with the protein nitrogen values of 94·1 (cf. Table 6), and 94·5%(43) which have been obtained by precipitation methods.

Estimation of free amino-nitrogen by the Van Slyke method

For determination of the free amino-nitrogen content of the stored powders a 4 ml. aliquot of the dialysed, reconstituted sample, prepared as described above (p. 322), was transferred to the reaction chamber of the manometric Van Slyke apparatus and allowed to react with the nitrous acid reagent for 30 min. at a temperature of 20° C. in the presence of capryl alcohol as defoaming agent. An exposure of 30 min. at 20° C. is not sufficient to complete the reaction and the 30 min. values have therefore been corrected by the addition of 2·0 units. Experiments on the application of the Van Slyke method to determination of the free amino-nitrogen content of casein and of fresh and deteriorated milk protein which form the basis for the above correction are described elsewhere (44). Since the correction is applied equally to all values, estimation of the *loss* of free amino-nitrogen which occurs during storage is the same whether the 30 min. or the corrected values be used. All data are reported as mg. free amino-nitrogen per g. undialysable ('protein') nitrogen.

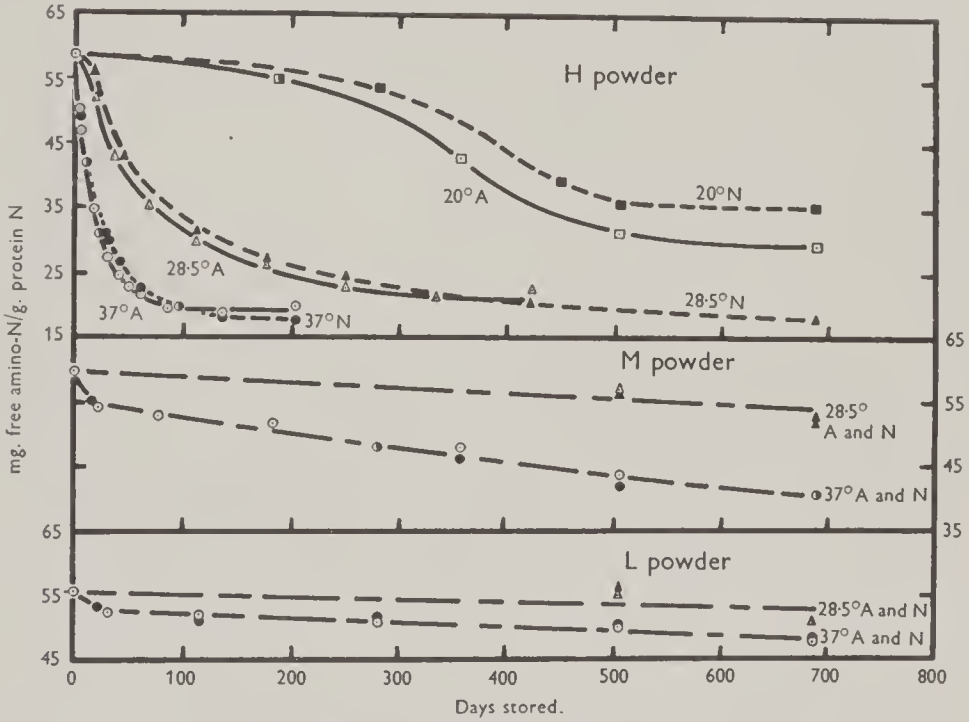

Fig. 17. Loss of free amino-nitrogen by the protein of the stored powders, as determined by the Van Slyke method.

Results

The results which are summarized in Fig. 17 and Table 7 show very marked losses in the free amino-groups of the protein during the storage of milk powder, the change once again being very greatly accelerated by a high moisture content in the powder, particularly when the storage temperature is also high. Evidence of an acceleration produced by the increase in equilibrium relative humidity resulting from crystallization of the lactose is again present in the data for H powder. Slight differences apparent between the rates of deterioration of H powder packed in air and in nitrogen are not greater than can be accounted for by the influence of the atmosphere on the crystallization of lactose at the several storage temperatures (p. 305), and no such effect is noticeable with M and L powders in which the lactose does not crystallize. The reaction responsible for disappearance of the free amino-nitrogen therefore does not require the presence of atmospheric

oxygen. The fact that the reaction comes to a stop, or at least slows down to a very low rate, when only about 70% of the original content of amino-nitrogen has been destroyed will be considered, in conjunction with other relevant data in the general discussion (p. 331).

A point of interest in connexion with the data for M and L powders at 37° C. is the rapid initial loss of 3–6 units of amino-nitrogen which appears to take place early in storage, a change which has no parallel in most of the other criteria determined, but is detectable also in the combined sugar content of the protein (Fig. 18). This point also will be referred to again in the discussion (p. 331).

Table 7. *Loss of free amino-nitrogen (Van Slyke) on storage of skim-milk powder**

		Time taken to lose the stated percentage of initial free amino-nitrogen content (days)							
		Air-pack				Nitrogen-pack			
Powder	Storage temp. (° C.)	5%	10%	20%	50%	5%	10%	20%	50%
H	37	2	3	5	25	2	3	6	30
	28·5	13	17	25	120	17	21	31	140
	20	130	230	320	650	130	300	380	—
M	37	10	40	320	—	10	40	320	—
	28·5	500	—	—	—	500	—	—	—
	20	—	—	—	—	—	—	—	—
L	37	25	—	—	—	25	—	—	—
	28·5	—	—	—	—	—	—	—	—

* Absence of a figure in the table signifies a period of over 700 days.

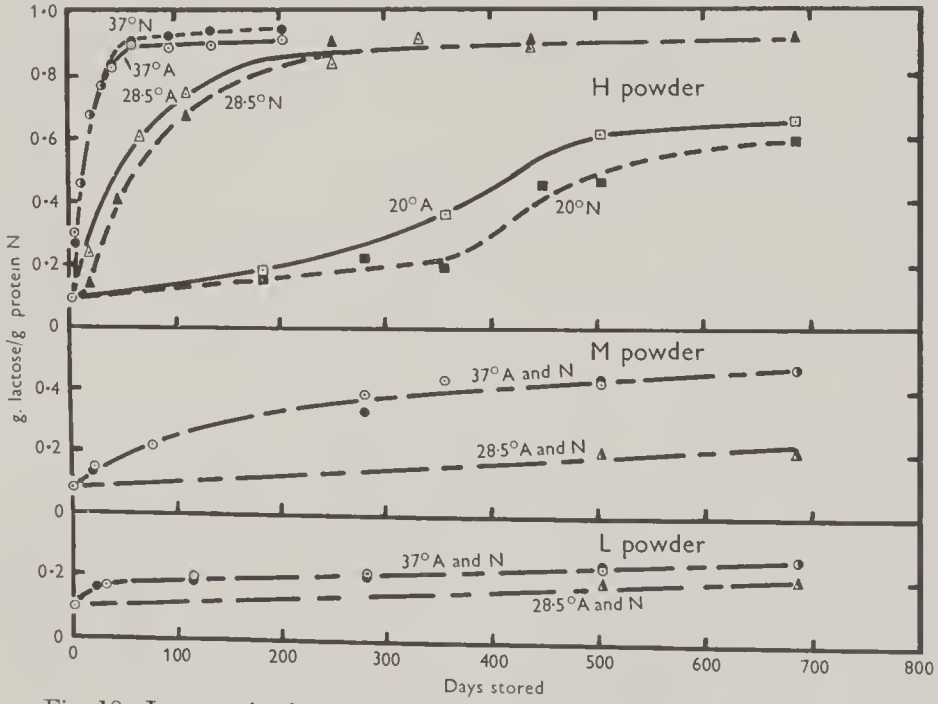

Fig. 18. Increase in the amount of sugar combined with the milk protein, as determined by the direct method.

Estimation of combined sugar

The increase in weight without a corresponding increase in the nitrogen content of the undialysable fraction of the milk, and the decrease in the free amino-content of the protein which accompanied deterioration of the powder on storage suggested that addition or

condensation of a sugar molecule or molecules was occurring at the free amino-groups of the protein. Since reaction with protein in such a way was likely to account for only a small proportion of the very large amount of carbohydrate present, some of which might in any case be destroyed by other reactions not involving protein, it was decided to attempt to follow the course of the protein-sugar reaction by direct estimation of the combined sugar rather than of the residual free sugar. Preliminary attempts to apply a colorimetric orcinol method were disappointing, and attention was directed towards the copper reagent recently described by Somogyi (45).

While any method depending on measurement of reducing power is open to some criticism on the grounds of lack of specificity, the copper reagent is known to oxidize sugars fairly selectively, and no great variety of interfering substances was likely to be encountered in dialysed separated milk. No information was available as to whether it would be possible to recover sugar quantitatively from combination with protein: the browning which accompanied serious deterioration suggested that some at least of the sugar was undergoing decomposition. It was decided therefore first to attempt estimation of the combined sugar *in situ*, without separation from the protein. Subsequent experiments were to be directed towards an investigation of the stability of the protein-sugar link, and examination of the fragments split off by hydrolysis.

Direct determination

The method of Somogyi involves heating 5 ml. of solution containing not more than 3 mg. of glucose or other sugar of equivalent reducing power with 5 ml. of the copper reagent in a freely boiling water-bath until reduction is complete. Lactose reacts comparatively slowly and a period of 30 min. was found preferable to the 20 min. specified by Somogyi for maltose. Glucose is completely oxidized in 10 min. After cooling, potassium iodide is added and the mixture is acidified and titrated with 0·005 N sodium thiosulphate. Determinations and blanks are run in triplicate. The calibration curve prepared from pure α-lactose hydrate is a straight line which very nearly passes through the origin. The presence of protein rendered observation of the end-point more difficult and appeared to cause slightly low recoveries of added sugar, but at the concentration required for the titration of deteriorated samples containing a reasonable amount of sugar such error was negligible.

Determination after acid hydrolysis

The Somogyi reagent is fairly strongly alkaline (pH 10), sufficiently so to be capable of causing some decomposition of the protein with liberation of ammonia and of inorganic sulphur and phosphorus when heated with it at 100° C. Combined sugar may therefore be split off during the determination. In case it should not and the result be too low duplicate determinations were carried out on several samples of powder after the dialysed milk suspension had been heated with N-HCl at 90° C. for 90 min. (or, alternatively, with 2 N-HCl at 100° C. for 60 min.), cooled and neutralized to pH 7. A calibration curve was prepared from pure α-lactose hydrate treated in the same way or, alternatively, from an equimolecular mixture of pure glucose and pure galactose. The presence of sodium chloride reduced the titre of sugar solutions, but the error was not serious at low salt concentrations and was corrected for by calibrating in the presence of suitable concentrations of sodium chloride. Within the limits of accuracy of the sugar deter-

minations the direct and the acid hydrolysis method both gave substantially the same result on the dialysed deteriorated milk powders, which suggests that the combined sugar was, in fact, mainly lactose, and that the reducing power of the sugar towards the alkaline copper reagent was not greatly affected by its union with protein.

Test of the validity of the method

To obtain more precise information as to the behaviour of combined sugar towards the Somogyi reagent protein-glucose and protein-lactose mixtures of known composition were stored at 37° C. and 55% relative humidity for periods up to several months. The results, which are reported elsewhere (8), show that although the estimation of combined sugar by the direct method in the presence of protein gives results which tend to be too low, the error should not be greater than about 10% when the concentration of sugar in the sample is reasonably high.

Results

Values obtained by the direct method on various of the stored milk powders are plotted in Fig. 18. The increase in combined sugar shows obvious signs of relationship with the loss of free amino-nitrogen (Fig. 17), and with the increase in weight of the undialysable fraction of the milk (Fig. 16). The quantitative aspect of this relationship will be considered later (p. 330).

The apparent sugar content of the protein from control powders and from fresh milk, and the possibility of the protein-sugar reaction commencing during processing

The undialysable fraction from fresh, control milk powders showed a small reducing power towards the Somogyi reagent, which could not be eliminated by any reasonable period of dialysis. Even the protein from fresh milk showed a definite, though apparently rather smaller, reducing power. Pending further investigations, it is not clear to what this initial reducing power of fresh milk protein is due, nor is information yet available as to how far the protein-sugar reaction is able to proceed during the normal commercial processes of precondensation, spray- and roller-drying, sterilization, etc.

SUBSIDIARY STORAGE EXPERIMENT (C. H. LEA)

In a previous section (p. 305) it has been shown that while the storage of L and M powders in sealed containers resulted in the maintenance of a reasonably uniform activity of water throughout the experiment, the crystallization of lactose in H powder caused an increase in the activity of water from 41–43% to about 55% relative humidity, a change which began after a noticeable induction period and only reached completion some 3–4, 30–40 and 300–400 days after the commencement of storage at 37, 28·5 and 20° C. respectively. Chemical changes in the powder of highest moisture content did not, therefore, take place at a uniform activity of water.

In order to compare the rates of deterioration at activities of water corresponding to the condition of M powder, which does not crystallize on storage, and of H powder in a sealed container before and after crystallization of the lactose, small samples of M powder were stored in a thin layer at 37° C. over solutions of sulphuric acid giving relative humidities of 29, 43 and 55%. Deterioration was followed by estimation of free amino-nitrogen by the Van Slyke technique, carried out in this case on the powder itself without

dialysis. The results, expressed as free amino-nitrogen on the total nitrogen basis, have been plotted in Fig. 19 as percentages of the initial value.

It is apparent from these data that the initial combination of protein and sugar would have proceeded fairly rapidly in the high moisture powder at 37° C. even if the lactose had failed to crystallize. At 20° C., on the other hand, the difference between the rates of deterioration of H powder before and after crystallization of the lactose was obviously greater, and the effect of crystallization can be traced in the record of most of the criteria of quality measured during the main storage experiment (cf. Figs. 1, 7, 8, 13, 16–18).

The rapid loss of about 10% of the initial content of free amino-groups during the first 20 days of storage at 29% R.H. confirms the similar observation already made on M powder at 37° C. in the main storage experiment (cf. Fig. 17, p. 325).

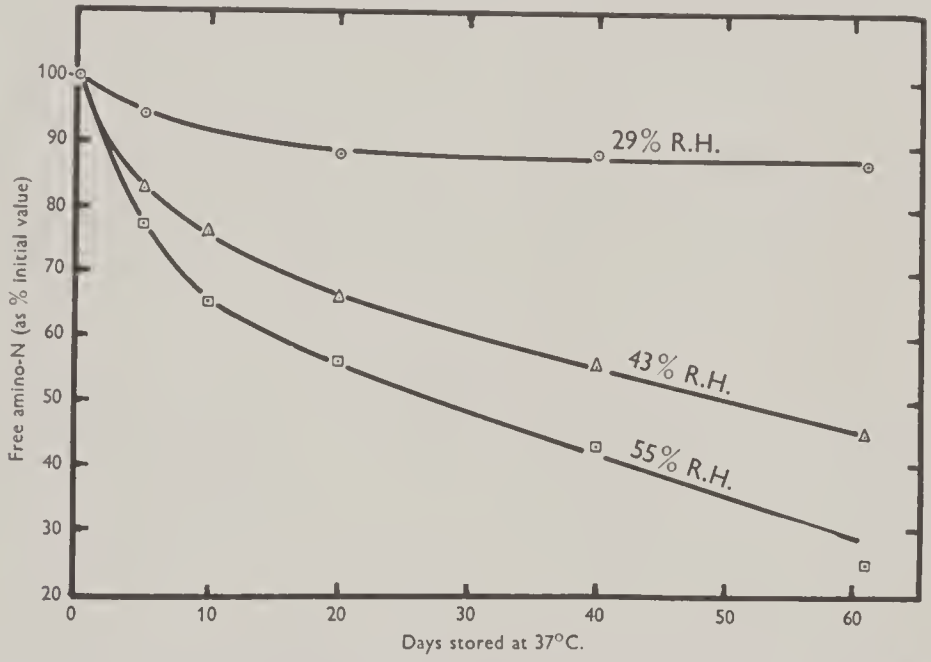

Fig. 19. Loss of free amino-nitrogen by separated milk powder during storage at 37° C. in atmospheres of constant relative humidity.

Bacteriological examination of the stored powders (J. C. D. White)

It is generally recognized and has been shown by Higginbottom (46) that the plate counts of both roller- and spray-dried milk powders tend to decrease during storage. Many samples, however, show little or no change in bacterial count after 6 months or longer. As the present experiment made available three skim-milk powders of different moisture contents which had been stored in air and nitrogen at three temperatures, the opportunity was taken to make a bacteriological examination of the powders after a long period of storage. Of special interest was the possible influence of the different equilibrium relative humidities on the survival of the bacteria. As shown on p. 304, the L and M powders remained at their initial equilibrium relative humidities of 17·5 and 29% respectively throughout storage. On the other hand, the equilibrium R.H. of H powder rose from its initial value of *c.* 42 to *c.* 55% after varying periods which depended on the storage temperature and atmosphere (cf. Fig. 5).

No thorough investigation was attempted of any changes in the bacterial count of the powders after varying lengths of time but the arbitrary period of 600 days' storage was chosen. The H powder stored at 28·5 and 37° C. was not examined since the complete

insolubility of the protein would make a plate count difficult and any result obtained of doubtful value. The plate counts were made by Dr C. Higginbottom using the same technique as for the fresh powders (cf. Part II, p. 295). The results are recorded in Table 8.

Table 8. *The bacterial count of the powders after storage for* 600 *days* (*the counts of the fresh powders are included for comparison*)

Powder	Storage temp. (° C.)	Pack	Plate count/g. powder	
			3 days at 37° C.	5 days at 30° C.
H	20	Air	104,000	224,000
		Nitrogen	264,000	—
Fresh	—	—	132,500	239,500
M	37	Air	171,000	75,000
		Nitrogen	89,000	98,000
	28·5	Air	94,000	79,000
		Nitrogen	148,000	172,000
	20	Air	122,000	151,000
		Nitrogen	232,000	244,000
Fresh	—	—	180,000	281,000
L	37	Air	179,000	187,000
		Nitrogen	246,000	249,000
	28·5	Air	232,500	246,000
		Nitrogen	274,000	249,000
	20	Air	255,000	238,000
		Nitrogen	228,500	256,500
Fresh	—	—	186,000	290,000

Although no definite conclusions can be drawn from the limited data available, the counts indicate a *tendency* to a higher survival rate with decreasing humidity, in nitrogen compared with air, and with decreasing storage temperature. Similar tendencies have been shown by Nichols(47) in respect of storage temperature and nitrogen-packing. Since the apparent increases in some of the plate counts (3 days at 37° C.) are within the experimental error, it seems that even the abnormally high equilibrium R.H. of H powder was insufficient to permit bacterial multiplication.

DISCUSSION

The protein-sugar reaction

The increase in weight of the undialysable (protein) fraction of the milk, together with the accompanying decrease in its free amino-nitrogen content and increase in its combined sugar suggest that, under suitable conditions, protein and reducing sugar react during the storage of milk powder to produce a non-dialysable substance, presumably an addition or condensation product formed by reaction between free amino-groups of the protein and potential aldehyde groups of the reducing sugar.

The relationship between the observed increase in weight of the undialysable fraction of the milk and the increase in its combined sugar content, as determined by direct estimation, is shown in Fig. 20A. The corresponding relationship between the increase in combined sugar and the decrease in free amino-(Van Slyke)-nitrogen content of the protein is given in Fig. 20B. The correlation coefficients in the two cases are 0·94 and 0·99 respectively. These observations cover a range of reaction velocities of the order of several hundreds to one, although observations are naturally limited to the earlier stages of the reaction for those conditions of moisture content and storage temperature which

permitted only relatively slight deterioration during the 2 years of the experimental period.

The deviation between the regression and the theoretical lines drawn in Fig. 20 B shows that approximately 0·88 molecule of lactose was estimated per free amino-group destroyed, the best individual values (gas-stored samples of H powder after 30–60 days at 37° C., or *c.* 250 days at 28·5° C.) giving a ratio of approximately 0·93. Since the direct method used for the estimation of sugar is liable to give results which are too low in about the

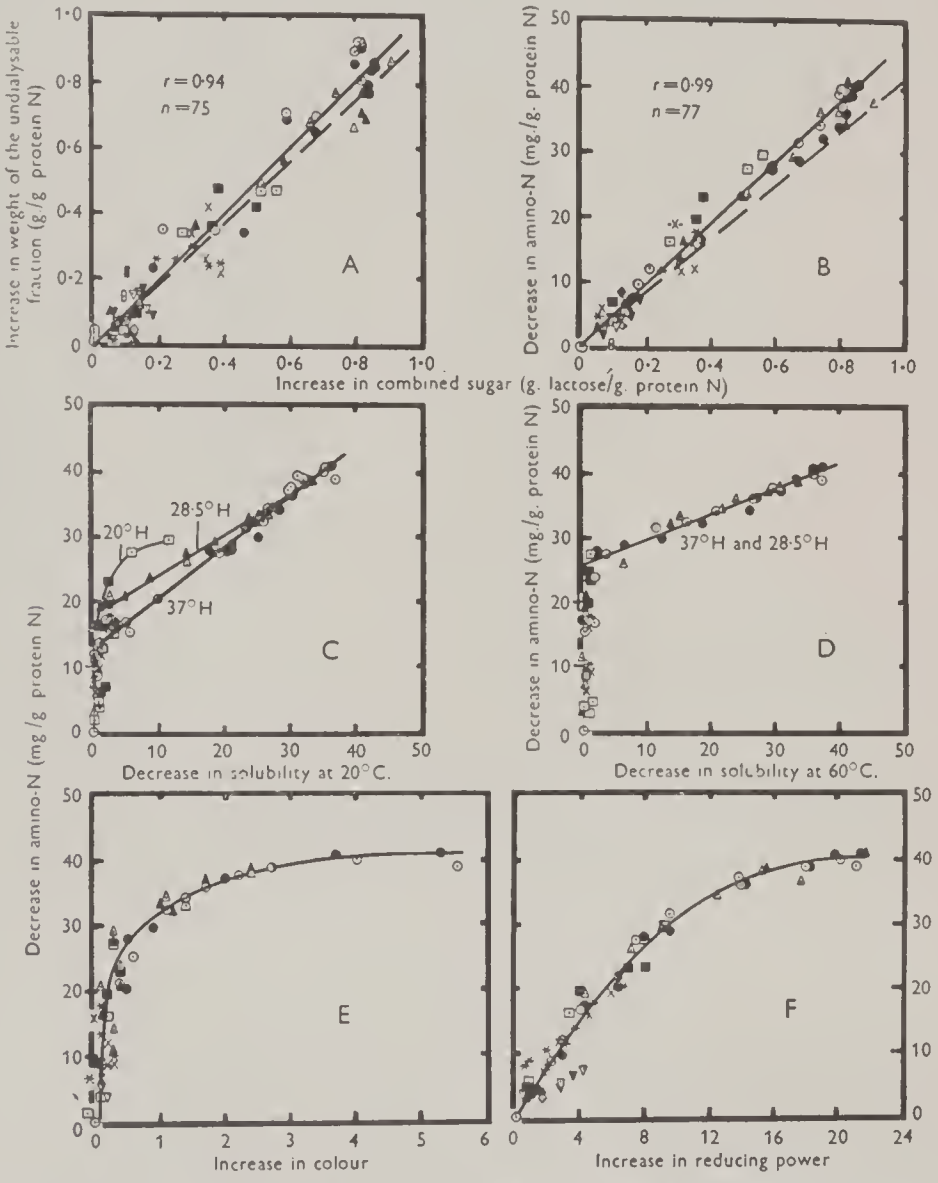

Fig. 20. Interrelations between various of the chemical criteria studied (Cambridge results).*

same ratio (p. 327) it can be seen that the loss of free amino-nitrogen and the increase in combined sugar are in fair agreement.

The slope of the theoretical line drawn in Fig. 20 A to represent the addition of a residue of equivalent mass 324 g. titrating as lactose is sufficiently close to that of the regression line for the experimental points to suggest that the reaction involved is, in fact, essentially the addition of lactose (mol. wt. 342) to the protein, probably with the

* The symbols used in Figs. 20–22 denote different conditions of storage, as follows: H powder 37° C. = ⊙, 28·5° C. = △, 20° C. = □; M powder 37° C. = ×, 28·5° C. = ◇, 20° C. = ⅄; L powder 37° C. = ▽, 28·5° C. = ⊡, 20° C. = ⊡. Where not otherwise indicated storage in nitrogen is shown by a 'solid' symbol.

elimination of a molecule of water (mol. wt. 18). An increase in the sugar values by about 10% would close the gap between the observed and calculated lines.

Despite the poor reproducibility of individual determinations of combined sugar or of increase in undialysable solids the general picture is therefore consistent with the combination of one molecule of lactose with each free amino-group destroyed, part of the combined sugar subsequently undergoing degradation on continued storage, particularly in air.

The relatively rapid drop of 3–6 units in free amino-nitrogen content which occurred early in the storage of M and L powders at 37° C. as well as in the subsidiary storage experiment (p. 328), to be followed by a much slower and linear change, is of interest in that it suggests the presence of a small amount of reactive material which might be either a small proportion of protein amino-groups of greater than average activity or accessibility or a small proportion of a reducing sugar more reactive than lactose. The change is, in fact, of the order of magnitude of that which might be produced by traces of glucose such as have been reported in milk (48, 49), and it is shown elsewhere that glucose, in fact, reacts with protein amino-groups considerably more rapidly than lactose. On the other hand, the reaction of a small amount of glucose appears to be largely suppressed in the presence of a big excess of lactose (8), and variable accessibility of the reacting groups to one another in the solid system is perhaps the more likely explanation.

The sugar-amino-group reaction in H powder at the higher storage temperatures, after a rapid start came virtually to a stop while some 30% of the free amino-nitrogen as determined by the Van Slyke method still remained undestroyed (Fig. 17). While some of the free amino-groups which do not react with reducing sugar may be different, chemically, from those which do (the free α-amino-group of lysine, for example, is known to be much less reactive towards formaldehyde than is the ε-amino-group (50)), it is, nevertheless, true that the ε-amino-groups of the lysine residues probably account for at least 90% of the total free amino-groups present. It appears therefore that some of these lysine amino-groups may be less favourably placed for reaction with sugar molecules, either through being folded within the protein molecule or simply because of the heterogeneity of the solid protein-sugar mass. On the other hand, it is possible that the establishment of an equilibrium between forward and reverse reactions is involved. Other cases in which reactions involving protein amino-groups stop short of completion have been reported. Thus, in the tanning of casein by formaldehyde in the cold under a variety of conditions only about two-thirds of the total Van Slyke free amino-nitrogen is destroyed (51). Similarly, in the production of toxoid from toxin by the action of weak formaldehyde over a long period the reaction ceases when part only of the free amino-nitrogen has been destroyed.

Comparison of the formol and Van Slyke data for free amino-nitrogen

It has already been pointed out that the formol titration of fresh milk powder or of the dialysed milk-protein fraction separated from it, gave a value for free amino-nitrogen in approximate agreement with the lysine content of the milk as found by microbiological assay (Part V), although perhaps very slightly below values which have recently been reported in the literature for milk powder and for casein. Kekwick & Cannan's (40) view that terminal α-amino-groups should not influence the determination to any great extent has also been quoted (p. 319).

The initial Van Slyke values obtained for two samples of fresh milk and for the L, M and H powders corresponded to 10·7, 11·1, 11·1, 11·9 and 11·7% of lysine in the milk

protein, calculated on the nitrogen basis: M and H powders were prepared from the sam batch of milk. Milk protein, though obviously variable in composition, usually contain an appreciably higher proportion of lysine than its major constituent casein, for which th best value is probably about 10·1% on the nitrogen or 8·3% on the weight basis (52). Th difference of 0·6–1·8% must therefore cover the higher lysine content of the non-casei proteins,* as well as any terminal free α-amino-groups present. Obviously, if the Va Slyke method is yielding results on the fresh protein which are too high, the error canno be very great.

Both formol and Van Slyke methods therefore yield reasonable initial values on th fresh-milk powders. The Van Slyke method, supported by the dry weight of the un dialysable fraction and by the combined sugar determinations, however, indicates a muc greater loss of free amino-groups (and hence of lysine) from the protein during storag than the formol titration method. Moreover, the shape of the curve relating loss of amino group to time of storage is also different for the two methods, a greater proportion of the tota loss occurring during the early part of storage when measured by the Van Slyke method

Microbiological assay (cf. Part V) on H powder after enzymic hydrolysis indicated a los of lysine during storage for 3 months at 37° C. of the order of 40%, which is greater tha the loss of free amino-groups found by the formol titration (15–20%), but less than tha found by the Van Slyke method (*c.* 65%). Microbiological assay after acid hydrolysi showed a smaller loss of lysine of the order of 15%.

It is difficult to imagine that the Van Slyke method should yield results on deteriorate powder which are too low, although it is by no means difficult to visualize partial re generation of lysine during hydrolysis of the protein, particularly with acid, prior t microbiological assay. The reason for the lack of agreement between the formol and Va Slyke determinations on deteriorated powder, however, is not yet clear. It is not due t regeneration of free amino-groups during the formol titration since titration of deteriorate powder to pH 8·5 produces no increase in Van Slyke nitrogen. One possibility that ha not yet been investigated is that the basic character of the amino-group is not sufficientl weakened by the first reaction it undergoes during deterioration of the powder to preven partial titration under the conditions of the formol estimations. Whatever the reason fo the discrepancy there seems little doubt, from the balance of the evidence available, tha the Van Slyke method gives the more useful picture of the changes occurring in the fre amino-groups of the protein during the storage of milk powder. Similar evidence has als been obtained for an artificial protein-glucose system (8).

The possible nature of the mechanism whereby the interaction of protein amino-group and reducing sugar apparently leads to the production of undesirable physical and chemica changes in both protein and sugar, and to the loss of biological value of the protein i discussed elsewhere (Part VI, p. 359 (8)).

Solubility

The relationship between the protein-sugar reaction, as measured by the decrease in free amino-(Van Slyke)-nitrogen, and the solubility of the powder, as determined after reconstitution in water at 20 or at 60° C., is shown in Figs. 20C and D. According to both

* Block & Mitchell (53), for example, quote 7·9, 9·6 and 11·4% for the lysine contents (weight basis) o casein, lactalbumin and β-lactoglobulin respectively, although they also give the obsolete value of 7·5% for the total protein.

sets of data a very considerable loss of free amino-nitrogen occurred before the onset of loss of solubility, the figures at 60° C. indicating that as much as two-thirds to three-quarters of the reactive amino-nitrogen, or nearly half of the total amino-nitrogen, was destroyed before solubility was appreciably reduced. Loss of solubility in water at 20° C., which apparently represents an earlier stage in deterioration, commenced at somewhat lower levels of amino-group loss, particularly when the powder had been held at high storage temperatures, but the 'lag' period was still very marked. This delayed onset of deterioration in solubility in relation to loss of free amino-nitrogen has also been demonstrated in experiments with artificial protein-sugar systems(8). It follows from these results that a considerable proportion of the free amino-groups of the protein molecule can combine with reducing sugar without appreciably affecting the solubility of the protein.

The question still remains whether insolubility is finally produced (*a*) simply by combination of still further amino-groups each with a molecule of reducing sugar, or (*b*) is the result of an induced denaturation of the protein molecule, or of degradation of the protein-sugar complex by secondary reactions between the now adjacent protein molecules and carbohydrate chains. The fact that a very small quantity of glucose, able to combine only with about one-eighth of the free amino-groups of the protein can, under suitable conditions, give rise to serious discoloration and insolubility of over two-thirds of the protein, this deterioration occurring after completion of the fall in amino-nitrogen(8), strongly supports hypothesis (*b*), as does evidence derived from the measurement of discoloration, reducing powder, evolution of carbon dioxide, etc., in the main storage experiment. The initiation of loss of solubility in cold water at progressively lower values of amino-nitrogen loss as the temperature of storage of the powder increases (Fig. 20C) is also suggestive of such a mechanism.

It has already been pointed out (p. 313) that the fresh powders differed appreciably from fresh milk in possessing a lower lactalbumin plus lactoglobulin, and a slightly higher apparent casein and proteose-peptone content, these changes presumably being a result of the pre-heating of the liquid milk. As deterioration progressed during storage, most of the casein and probably most of the lactalbumin became insoluble, while the lactoglobulin appeared to remain soluble until an extreme stage of deterioration had been reached. A small amount of proteose-peptone was produced, of which part at least arose from decomposition of the casein, but the absence of any increase in non-protein nitrogen indicated that no drastic fragmentation of the protein molecule had occurred.

Colour

The nature of the relationship between the protein-sugar reaction and discoloration of the powder is shown in Fig. 20E. As with solubility in hot water about three-quarters of the reactive or one-half of the total free amino-nitrogen disappears, presumably as the result of combination with sugar, before any very marked change in colour occurs. Even when the primary sugar-amino-group reaction has almost ceased the development of discoloration proceeds at an almost undiminished rate. Apparently the protein-sugar complex first formed is not coloured, but becomes so on degradation. The experiments in which dialysed milk protein was stored in presence or absence of added reducing sugar have shown that the complex is unstable under conditions which cause no discoloration of protein or sugar alone(8). The possibility that discoloration may be due in part to the caramelization of lactose catalysed by amino-nitrogen is discussed elsewhere(8).

Reducing power

The increase in reducing power of the whole powder towards ferricyanide, as measured by the Chapman & McFarlane(25) method shows a good correlation with the progress of the primary sugar-amino reaction under the various storage conditions (cf. Figs. 13, 16–18) until about three-quarters of the reactive or one-half of the total free amino-nitrogen has reacted with sugar (Fig. 20F). This behaviour is consistent with the observation that neither protein nor sugar alone develops on storage the power of reducing ferricyanide under conditions which cause the mixture (complex) rapidly to do so(26).

For powders stored at 37 and 28·5° but apparently not at 20° C., the reducing power of the milk continues to increase, after the primary amino-group-sugar reaction has almost ceased, thus suggesting more extensive decomposition of the protein-sugar complex at the higher storage temperatures. The data on colour and weight increase (Figs. 3 and 16) have already indicated more extensive decomposition of the protein-sugar complex under such conditions. It has been shown elsewhere that soluble, dialysable, non-nitrogenous substances, which are presumably degradation products of the protein-sugar complex or caramelized sugar, contribute to the reducing power of powder of high moisture content stored for long periods at 37° C.(26).

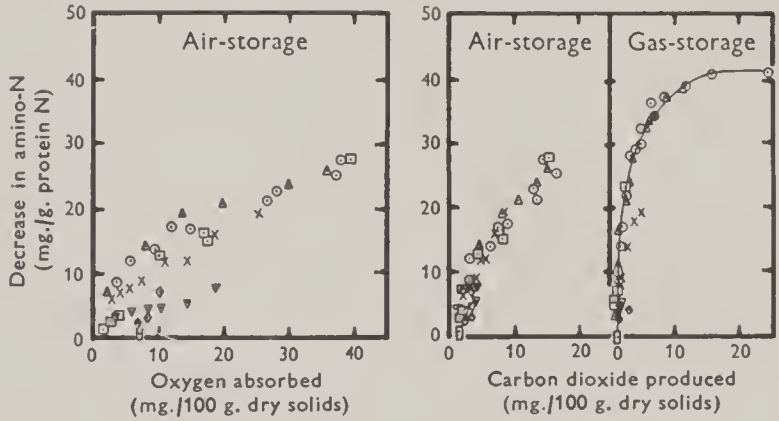

Fig. 21. Relations between free amino-nitrogen and gas exchange (Cambridge results).

Absorption of oxygen and production of carbon dioxide

Production of carbon dioxide by the several gas-packed powders at the several storage temperatures increased sufficiently in step with the decrease in free amino-nitrogen content of the protein to suggest that degradation of the protein-sugar complex was the main source of carbon dioxide production by the gas-packed material (Fig. 21). This process continued after formation of the protein-sugar complex had ceased. Under conditions which inhibited the protein-sugar reaction almost no carbon dioxide was produced by gas-packed, and very little by air-packed, powder (Table 4).

In the air-packed cans the production of carbon dioxide was much more rapid (until all free oxygen had been absorbed after which the rate fell to that of the gas-packed material), but appeared still to be largely derived from the protein-sugar complex (Fig. 21). Absorption of oxygen by the air-packed materials, however, showed a less obvious connexion with loss of amino-nitrogen. The powder of high moisture content absorbed oxygen roughly in proportion to the progress of the amino-group-sugar reaction, indicating that the oxygen was probably being used largely in degradation of the protein-sugar complex. With M powder at 37° C., and particularly with materials such as L

powder at 37° C., or M powder at 28·5° C., in which the protein-sugar reaction was very slow, however, a relatively too rapid absorption of oxygen over the long period of the test suggests the existence of another system slowly absorbing oxygen, with but little production of carbon dioxide. Such a system might perhaps be provided by fat, of which a small proportion was still present in these 'separated' milk powders. This point will be considered again later in connexion with deterioration in flavour.

The amount of complete degradation to carbon dioxide and water which occurred was comparatively small, the maximum amount of carbon dioxide liberated by the air-packed H powder during storage for 203 days at 37° C., for example, corresponding to rather less than 1% of the weight of sugar bound by the protein, or less than 0·05% of the weight of the protein-sugar complex.

Flavour

A notable feature of the taste panel results was the extreme rapidity with which 'off'-flavour developed under the more unfavourable conditions of storage; H powder, for example, passed below the acceptance level after about 2 days in air at 37° C. On the other hand, gas-packed M and L powders at 20° C. were still well above the acceptance line after 2 years (Table 1).

Since the senses of taste and smell are frequently affected by amounts of chemical change which appear small when measured by ordinary chemical means, the rapidity with which serious 'off'-flavours developed in some of the experimental powders does not in itself disprove any connexion between taste and the chemical criteria measured. There is, in fact, when due allowance is made for the wide range of stabilities encountered and for inaccuracies inherent in the tasting technique, a general parallelism between flavour deterioration and a number of the chemical criteria, a parallelism which improves as the range of experimental conditions included in the survey is restricted.

The most obvious discrepancy between the tasting data and chemical tests based directly on the protein-sugar reaction is that of the relative keeping properties of the powders when packed in air and in nitrogen. As already pointed out (p. 324) there is no evidence that the rate of combination of protein amino-groups and reducing sugar is influenced by the atmosphere in the container, except indirectly as a result of changes in the activity of water resulting from the crystallization of lactose. This occurred only in H powder, and the effect was generally quite small, being greatest for storage at 20° C. Chemical tests based on secondary changes involving the protein-sugar reaction, e.g. loss of solubility and discoloration, also seemed to be affected only slightly by packing in nitrogen instead of in air, and here again part at least of the protective effect of gas-packing could be attributed to delayed crystallization of the lactose in H powder.

On the other hand, differences between the rates of deterioration of flavour in air- and inert gas-pack were very marked indeed for all three powders at all three storage temperatures, and M and L powders at least, showed no difference in the rates of combination of protein and sugar in air and in nitrogen to account for the more rapid development of 'off'-flavour in air-storage (Figs. 16–18).

When deterioration in flavour is plotted against decrease in free amino-nitrogen the correlation is found to be quite good for the gas-stored, but less so for the air-stored samples (Fig. 22 A). It appears that, while the protein-sugar reaction is probably fairly directly responsible for the deterioration in flavour of gas-stored powders, in air a

considerable additional development of 'off'-flavour occurs, and is present even under conditions which permit little or no reaction between protein and sugar.

Production of carbon dioxide by the gas-stored samples also shows a good correlation with deterioration in flavour (Fig. 22 B), and is presumably a measure of the thermal or spontaneous degradation of the protein-sugar complex (or, possibly, of protein-catalysed caramelization of sugar), which in turn probably accounts for the development of 'off'-flavour and colour in the gas-stored powders. As previously pointed out, 'off'-flavour in gas-stored samples has frequently a predominantly 'caramelized' or 'burnt-sugar' character.

Absorption of oxygen by the air-stored samples, which also shows some parallelism with deterioration in flavour, is probably accounted for partly by oxidation of the decomposing protein-sugar compound with evolution of much carbon dioxide, and partly

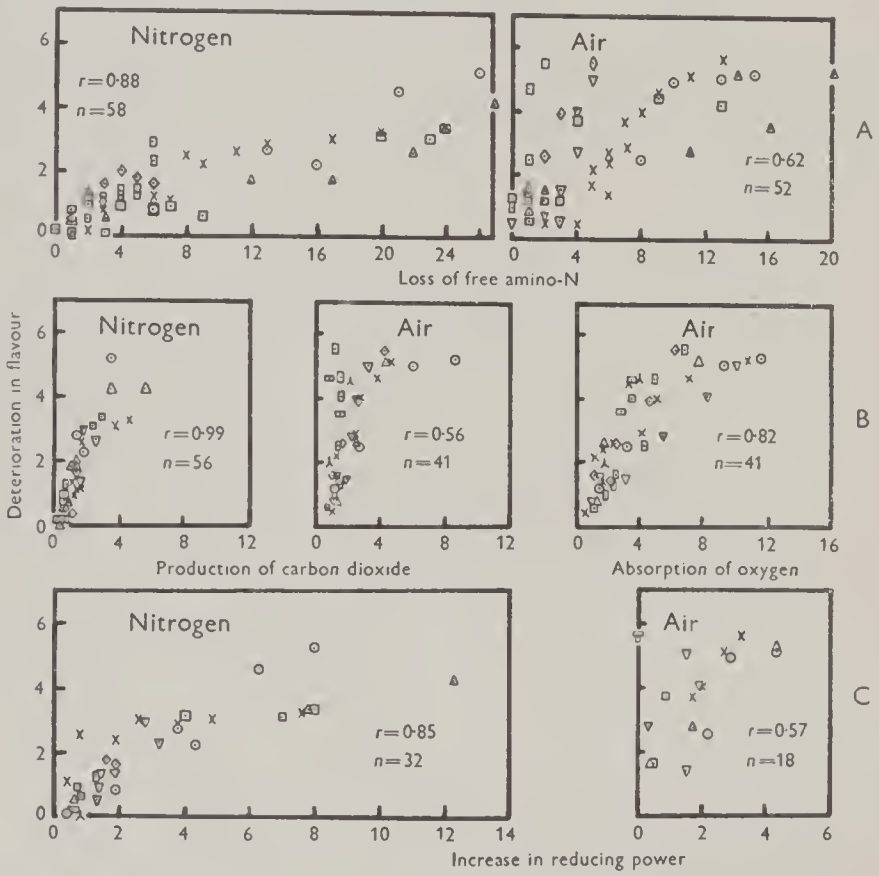

Fig. 22. Relations between flavour and various of the chemical criteria (Cambridge results).

by oxidation of some other component of the system (possibly fat) with evolution of relatively little carbon dioxide. The former mechanism is of major importance for samples of high, and the latter for samples of low, moisture content. The odour and flavour developed by powders of high moisture content deteriorating in air have a pronounced 'stale and glue-like' character.

The reducing power of the powders towards potassium ferricyanide shows a fair degree of correlation with deterioration in flavour (Fig. 22 C), but again the relationship between reducing power and flavour varies appreciably with storage conditions, and particularly with the atmosphere in the container: whether the gas in the can be air or nitrogen reducing power develops at approximately the same rate, while 'off'-flavour appears more rapidly in air.

To summarize, it appears that the failure of the chemical tests to co-ordinate more successfully with deterioration in flavour over a wide range of moisture content of the powder and conditions of storage is probably due to the fact that 'off'-flavour as assessed by the panel is not a simple phenomenon resulting from one reaction, but is a complex built up from a number of components resulting from several types of reaction.

These include (*a*) anaerobic degradation of the protein sugar-complex (and perhaps, the protein-catalysed caramelization of sugar) as found in gas-stored powders of high moisture content, (*b*) oxidation of the protein-sugar complex or of its degradation products (and, perhaps of caramelized sugar), proceeding together with (*a*) in air-stored powders of high moisture content, and (*c*) oxidation of some constituent or constituents of the powder, possibly fat, which proceeds independently of the protein-sugar reaction and is mainly of importance in air-packed powders of low moisture content stored for long periods.

Since different small groups of tasters cannot be expected to attach precisely the same relative significance to 'off'-flavours of several types, some divergence of opinion between panels as to the effects on palatability of the various storage factors studied in the present work is inevitable. Comparison of the results given by the panels operating independently at Cambridge and at the Hannah Institute, however, shows that the effect of such differences of opinion, as well as of an overall difference in severity of marking, has not been sufficient to obscure a substantial measure of agreement on the main points at issue (Table 1).

Effect of moisture content of the powder, of storage temperature and of atmosphere in the container on the rate of deterioration

Consideration of the data accumulated during storage of the experimental powders at three levels of moisture content, at three storage temperatures and in two atmospheres brings out many points of similarity. By all criteria the moisture content of the powder was the factor of greatest importance in determining storage life, the L (3·0%) and M (5·0%) powders being relatively stable, with a definite advantage in favour of the former, while the H (7·6%) powder was very unstable. Part of the large difference in behaviour between H powder and the others can be attributed to the fact that the α-lactose originally present in a 'glassy' supersaturated form crystallized in H powder (the process requiring a few days at 37°, a few weeks at 28·5° and a few months at 20° C.), thereby increasing the activity of the water in the residual protein-sugar mixture and accentuating the difference between the high moisture and the other powders, in which crystallization did not occur even after many months at 37° C.

The temperature coefficient of deterioration of H powder was also very high, the rate usually being increased by a factor of at least 5 for each 8·5° C. increase in temperature, corresponding to a Q_{10} of at least 6. This extreme sensitivity to slight variation in moisture content in the upper part of the range tested, and to slight differences in storage temperature seemed to be a characteristic of the protein-sugar reaction. The effect of change in moisture content was smaller and the temperature coefficients much lower (Q_{10} of the order of 2) when change could be detected, e.g. in flavour or in gas exchange, under conditions in which but little reaction between protein and sugar occurred (e.g. M powder at 28·5 or 20° C., and L powder at 37, 28·5 and 20° C.).

The probable connexion between delayed crystallization in gas-packed as compared

with air-packed H powder and the slightly slower protein-sugar reaction in the former material, particularly at 20° C., has already been pointed out, and a small amount of evidence produced suggesting that some cause other than slight fortuitous differences in moisture content was responsible. On the other hand, such small differences in moisture content were shown to account quite satisfactorily for the considerable 'scatter' in crystallization time among individual cans of air-packed H powder at 20° C. (cf. p. 306), and the question of the cause of the air-gas difference observed with this powder cannot be regarded as satisfactorily settled. The influence of storage atmosphere, however, on all criteria excepting only flavour and gas exchange, for which separate explanations are available, was comparatively small.

Summary

1. Three spray-dried separated milk powders with moisture contents of 2·9, 4·7 and 7·3% (3 hr. air oven) or 3·0, 5·0 and 7·6% (20 hr. vacuum oven) were packed in air and in almost pure nitrogen, in gas-tight cans, and stored at 20·0, 28·5 and 37° C., for a period of nearly 2 years.

2. The powders were examined at intervals for palatability, colour, pH, equilibrium relative humidity (indicating crystallization of the lactose), conversion of β-lactose to α-lactose hydrate, decrease in total soluble lactose, absorption of oxygen, production of carbon dioxide, solubility in water at 20 and at 50 or 60° C., changes in the distribution of soluble nitrogen, reducing power towards potassium ferricyanide, base-binding capacity, formol titration, weight of the undialysable fraction, free amino-nitrogen by the Van Slyke method, sugar attached to the protein, and bacterial content.

3. Little change was observed in the powders of low and medium moisture content except in palatability and gas exchange at the higher temperatures. The powder of the highest moisture content, particularly at the higher storage temperatures, rapidly became unpalatable, discoloured and insoluble. Its pH, free amino-nitrogen and soluble lactose content fell, the amount of sugar attached to the protein and the reducing power of the powder towards potassium ferricyanide increased. Oxygen was absorbed and carbon dioxide produced.

4. It was concluded that a major cause of deterioration in powder of high moisture content, particularly at high storage temperatures, is a reaction involving the free amino-groups of the milk protein, which will consist very largely of the ϵ-amino-groups of the lysine residues. The first stage of this reaction appears to be between the protein amino-groups and the potential aldehyde group of reducing sugar.

5. The rate of the protein-sugar reaction is largely determined by the moisture content or, more correctly, by the activity of moisture in the powder, and is very much greater at 7·6 than at 5·0 or 3·0%. Crystallization of lactose, which occurred only in the powder of highest moisture content, increased the activity of the residual water in the sealed container, and further accelerated deterioration.

6. The temperature coefficient of the formation (and degradation) of the protein-sugar complex is high (at least 6), and moisture contents which can be tolerated under temperate conditions for long periods will be unsatisfactory at high storage temperatures.

7. The reaction between protein and reducing sugar takes place in at least two stages, the primary combination resulting neither in discoloration nor in loss of solubility, which follows only as the result of secondary changes, the nature of which is not fully understood.

8. Those physical and chemical properties of the milk powders which depended essentially on the protein-sugar reaction were influenced only slightly by the nature of the atmosphere in the container.

9. The protein-sugar reaction resulted in the production of 'off'-flavours described as mainly 'heated' or 'caramelized' in the case of gas-packed powders, and as 'stale' and 'gluey' in the case of the air-packed powders. The latter were considered the more objectionable.

10. Evidence was also obtained of an oxidative reaction (or reactions), independent of the protein-sugar change, which produced 'off'-flavour in powders stored for long periods at moisture contents too low for the protein-sugar reaction to occur. It is possible that the small amount of fat present in the powders was involved.

11. These two factors resulted in a decided advantage for gas-packing, in so far as palatability was concerned, at all moisture contents and storage temperatures.

12. Owing to the complex nature of the causes of 'off'-flavour, no single chemical test correlated satisfactorily with the tasting panel score over all conditions of storage, the biggest discrepancy usually being experienced between air-packed samples on the one hand and gas-packed on the other. There was, however, a general parallelism between several of the chemical criteria measured and palatability, which was improved as the range of composition and storage conditions was narrowed.

13. Several of the chemical tests linked up fairly directly with the protein-sugar change, and of these the measurement of reducing power towards ferricyanide by the Chapman & McFarlane technique (25, 26) showed promise.

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PART IV. CHANGES IN THE BIOLOGICAL VALUE OF THE PROTEINS

BY KATHLEEN M. HENRY AND S. K. KON

(With 1 Figure)

The observations which have led to the inquiry now described are outlined in Part I of this report.

EXPERIMENTAL

General procedure

Animals and technique of tests

In most of the tests described below, the biological value (b.v.) and true digestibility (t.d.) of the proteins of the milk powders were measured on rats by the method of Mitchell (1, 2), and at the same time the protein efficiency (p.e.) (i.e. the gain in weight of the animal expressed in g./g. protein intake) was determined by the method of Osborne, Mendel & Ferry (3). The milk powders were included in the diets to supply approximately 8% protein (N × 6·38). Table 1 gives the composition of the experimental diets, Table 2 their analysis and a description of the milks tested in the various experiments.

In each test carried out by the balance-sheet method (1, 2) twelve female rats were used in litter-mate comparisons, and during the course of any one experiment all rats received each diet in turn. Full details of the experimental technique have been described by Henry, Kon & Watson (4). A maximum of four milk powders was tested simultaneously by this method. Male rats were used in the growth tests (3), also in litter-mate comparisons, each substance tested being offered to six rats. The animals received the experimental diets for 4 days in order that they might become accustomed to the change from the stock diet; subsequently records of weight increases and of diet intake were kept for a period of 4 weeks.

Milk powder used as a standard for comparison

It will be recalled that the H powder was prepared by raising the moisture content of a quantity of M powder whereas the L powder was made from a separate batch of liquid milk (cf. p. 294). Though there is no doubt that originally the three powders did not differ in the nutritive values of their proteins (cf. p. 347), M powder rather than L powder was taken as a standard for comparison; for this purpose cans of it, gas-packed and stored at −20° C. until needed, were used.

The selection of the powders examined in biological tests was guided by the results of chemical measurements (Part III).

Effect of the addition of cystine to samples of dried skim milk, freshly prepared, or stored for 5 years (Exp. 1)

It was originally thought (5) that cystine might be the amino-acid affected in the deterioration, on storage, of dried skim-milk powder. An experiment to test this view was, therefore, the first to be done, while the powders used in the main study were being made ready.

L-Cystine was added to the original deteriorated milk powder (6, 7), which by this time had been stored for 5 years, and to the freshly prepared low moisture powder (L control)

Table 1. *Percentage composition of the experimental diets*

Milk tested*	Diet no.	Milk	Salts†	Margarine fat	Sugar granulated ground	Potato stareh	Rice starch
Dried in 1939‡	257	21·7	4·0	10·0	12·0	10·0	42·3
Dried in 1939‡ + 0·18% cystine	258	21·9	4·0	10·0	12·0	10·0	42·1
L	259	22·0	4·0	10·0	12·0	10·0	42·0
L + 0·18% cystine	260	22·3	4·0	10·0	12·0	10·0	41·7
M	263§	23·2	4·0	10·0	12·0	10·0	40·8
H	267‖	23·7	4·0	10·0	12·0	10·0	40·3
Freeze-dried	273	22·6	4·0	10·0	12·0	10·0	41·4
Freeze-dried + 0·18% cystine	274	22·7	4·0	10·0	12·0	10·0	41·3
M + 2·5% lysine	282	22·4	4·0	10·0	12·0	10·0	41·6
H + 2·5% lysine	283	22·6	4·0	10·0	12·0	10·0	41·4
M + 1·25% lysine	284	22·9	4·0	10·0	12·0	10·0	41·1
M + 5% lysine	285	21·6	4·0	10·0	12·0	10·0	42·4
H + 1·25% lysine	286¶	23·4	4·0	10·0	12·0	10·0	40·6
M + 0·5% histidine + 0·5% arginine	290	22·2	4·0	10·0	12·0	10·0	41·8
H + 1·25% lysine + 0·5% histidine	291	23·0	4·0	10·0	12·0	10·0	41·0
H + 1·25% lysine + 0·5% histidine + 0·5% arginine	292	22·6	4·0	10·0	12·0	10·0	41·4

* Except where otherwise stated all samples of skim milk were spray-dried. L = low, M = medium, H = high content of moisture (cf. Part II).

† de Loureiro (1931). *Arch. Patol., Lisboa*, 3, 72. ‡ Tested in 1945. § 263 and 263 A.

‖ 267, 267 A, 267 B, 267 C, 267 D, 267 E, 267 F and 267 G. ¶ 286 and 286 A.

Table 2. *Analysis of the diets used in the various experiments*

Diet no.	Description of the dried skim milk tested*	% N	% moisture
	Exp. 1		
257	Spray-dried, prepared in 1939	1·280	9·02
258	Spray-dried, prepared in 1939 + 0·18% cystine	1·280	9·04
259	L control	1·272	7·98
260	L control + 0·18% cystine	1·277	8·18
	Exp. 2		
273	Freeze-dried	1·238	9·02
274	Freeze-dried + 0·18% cystine	1·242	9·15
	Exp. 3		
259	L control	1·269	8·22
263	M control	1·282	8·54
	Exp. 4		
267	H, air-packed, stored at 37° C. for 8 days	1·288	9·46
267 A	H, air-packed, stored at 37° C. for 28 days	1·297	9·06
267 B	H, gas-packed, stored at 37° C. for 28 days	1·282	9·39
267 C	H, air-packed, stored at 37° C. for 85 days	1·273	9·34
	Exp. 5		
263	M control	1·282	8·61
263 A	M, air-packed, stored at 37° C. for 182 days	1·277	9·02
267 D	H, air-packed, stored at 28·5° C. for 176 days	1·258	9·50
267 E	H, gas-packed, stored at 28·5° C. for 176 days	1·260	9·33
	Exp. 6		
263	M control	1·248	9·00
282	M control + 2·5% lysine	1·280	9·06
267 F	H, gas-packed, stored at 37° C. for 60 days	1·244	9·41
283	H, gas-packed, stored at 37° C. for 60 days + 2·5% lysine	1·259	9·33
	Exp. 7		
263	M control	1·269	9·87
284	M control + 1·25% lysine	1·272	9·64
282	M control + 2·5% lysine	1·270	9·58
285	M control + 5% lysine	1·267	9·95
267 F	H, gas-packed, stored at 37° C. for 60 days	1·267	9·73
286	H, gas-packed, stored at 37° C. for 60 days + 1·25% lysine	1·283	9·20
	Exp. 8		
263	M control	1·267	9·30
290	M control + 0·5% histidine + 0·5% arginine	1·272	9·06
267 G	H, air-packed, stored at 37° C. for 60 days	1·256	9·20
286 A	H, air-packed, stored at 37° C. for 60 days + 1·25% lysine	1·256	9·63
291	H, air-packed, stored at 37° C. for 60 days + 1·25% lysine + 0·5% histidine	1·265	9·33
292	H, air-packed, stored at 37° C. for 60 days + 1·25 lysine + 0·5% histidine + 0·5% arginine	1·268	9·43

* L = low, M = medium, H = high, content of moisture (cf. Part II).

which was intended for use in the main experiments. On the assumption that all the cystine of the deteriorated dried milk had been destroyed during storage, the amino-acid was added to both powders at the rate of 0·5% of the milk proteins. The b.v. and t.d. of these milks were determined by the balance-sheet method (1, 2), but a test by the rat-growth technique (3) was not included as there was not enough of the deteriorated milk left. The results of the experiment are given in Tables 3 and 5.

It will be seen that a statistically significant improvement in the b.v. from 90·4 to 95·2 was observed when cystine was added to the freshly prepared dried skim milk. On the other hand, no significant improvement resulted from the addition of cystine to the deteriorated sample, b.v.'s of 77·9 and 76·6 respectively being obtained with and without it.

Table 3. *Mean results for groups of* 12 *rats for the biological values and true digestibilities of the proteins* (N × 6·38) *of the milks studied in the various experiments*

				Biological value		True digestibility	
Exp. no.	Age of rats at start (days)	Weight of rats at start (g.)	Diet no.	Numerical result and its S.E. of the mean	Statistical significance*	Numerical result and its S.E. of the mean	Statistical significance*
1	26	56–57	257	76·6 ± 1·79	5% = 2·89 1% = 3·96 0·1% = 5·39	87·3 ± 0·69	5% = 2·61 1% = 3·58 0·1% = 4·87
			258	77·9 ± 1·82		86·1 ± 1·21	
			259	90·4 ± 2·09		92·4 ± 0·75	
			260	95·2 ± 1·63		95·0 ± 0·74	
2	25–26	43–57	273	90·1 ± 2·50	S.E.M. = 2·87 P = 1 : 34	92·5 ± 1·16	S.E.M. = 0·83 P = 1 : 29
			274	97·3 ± 1·04		94·5 ± 0·93	
4	24–25	54–58	267	86·9 ± 1·35	5% = 3·90 1% = 5·35 0·1% = 7·28	90·9 ± 0·57	5% = 2·46 1% = 3·37 0·1% = 4·59
			267 A	78·8 ± 1·49		89·4 ± 0·88	
			267 B	83·3 ± 1·79		89·6 ± 0·72	
			267 C	65·9 ± 1·79		85·6 ± 1·01	
5	23–25	46–58	263	83·5 ± 2·49	5% = 3·79 1% = 5·20 0·1% = 7·08	92·7 ± 0·73	5% = 1·61 1% = 2·20 0·1% = 3·00
			263 A	84·5 ± 1·51		89·4 ± 0·74	
			267 D	71·2 ± 1·99		87·8 ± 0·87	
			267 E	75·7 ± 1·31		88·2 ± 0·60	
6	25	54–61	263	84·5 ± 3·05	5% = 5·63 1% = 7·72 0·1% = 10·51	91·2 ± 0·70	5% = 1·85 1% = 2·53 0·1% = 3·45
			282	76·4 ± 2·69		91·4 ± 0·76	
			267 F	67·5 ± 1·22		86·0 ± 0·77	
			283	80·1 ± 2·43		89·0 ± 0·72	
7	23–24	49–61	263	87·6 ± 1·73	5% = 2·78 1% = 3·81 0·1% = 5·19	92·7 ± 0·66	5% = 1·99 1% = 2·72 0·1% = 3·71
			284	83·7 ± 1·70		91·6 ± 0·72	
			267 F	71·4 ± 0·93		85·1 ± 1·13	
			286	86·7 ± 1·29		87·0 ± 0·53	

* In all experiments except no. 2 an analysis of variance was carried out, for details see Table 5, the least mean differences necessary for significance at 5, 1 and 0·1% are quoted here. In Exp. 2 the paired *t*-test of 'Student' (*Biometrika*, 1908, **6**, 1; *Metron*, 1925, **5**, 105) was used. Comparisons were made between values obtained for each milk with the same rat. S.E.M. = standard error of the mean of the difference. *P* = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

The b.v. of 76·6 now obtained for the proteins of the deteriorated milk is higher than the previous value of 71·1 after $4\frac{1}{2}$ years' storage (6, 7). This can probably be attributed to variations in response between different batches of rats, and emphasizes the advisability of including a standard of comparison in such tests.

In previous tests there was no indication of a decrease in the t.d. of the milk whose b.v. decreased on storage (6, 7). In the present experiment the t.d. proved inferior to that of the L control powder. The latter, moreover, was further increased by the addition of cystine, whereas no such benefit was conferred on the deteriorated sample. Changes in

t.d. of powders on the addition of cystine or lysine were also observed in Exps. 2, 6 and 7 these findings are more fully considered in the discussion (p. 352).

Effect of the addition of cystine to freeze-dried milk (Exp. 2)

Fairbanks & Mitchell(8) found that roller-drying at the lowest possible temperature, or spray-drying without preheating, did not impair the b.v. of liquid skim milk and that the dried product was improved by the addition of cystine. They concluded from growth tests that cystine was affected by the heating but did not carry out tests with cystine added to liquid skim milk.

Table 4. *Mean results for groups of* 6 *rats for the protein* (N × 6·38) *efficiencies of the milks studied in the various experiments*

Exp. no.	Age of rats at start (days)	Weight of rats at start (g.)	Diet no.	Diet intake (g.)	Gain in weight (g.)	Protein efficiency: Numerical result and its S.E. of the mean	Protein efficiency: Statistical significance*
3	33–38	94–124	259	369·35	83·0	2·78 ± 0·075	S.E.M. ± 0·12
			263	376·77	90·3	2·93 ± 0·105	$P = 1:4$
4	22–25	46–73	267	289·21	67·5	2·83 ± 0·062	5% = 0·30
			267 A	264·24	50·0	2·28 ± 0·053	1% = 0·41
			267 B	271·52	53·8	2·38 ± 0·183	0·1% = 0·57
			267 C	225·10	26·0	1·41 ± 0·161	
5	24–26	48–68	263	285·03	64·8	2·79 ± 0·096	5% = 0·24
			263 A	261·56	54·7	2·56 ± 0·097	1% = 0·34
			267 D	258·23	43·5	2·10 ± 0·036	0·1% = 0·47
			267 E	272·15	49·2	2·24 ± 0·086	
6	25–26	63–69	263	273·82	61·2	2·81 ± 0·037	5% = 0·19
			282	262·98	55·8	2·59 ± 0·098	1% = 0·26
			267 F	251·89	36·3	1·82 ± 0·081	0·1% = 0·36
			283	279·01	57·3	2·55 ± 0·069	
7	24–26	51–73	263	284·55	62·5	2·71 ± 0·039	5% = 0·21
			284	286·36	62·8	2·70 ± 0·043	1% = 0·29
			282	293·77	62·2	2·60 ± 0·080	0·1% = 0·40
			285	282·62	53·8	2·35 ± 0·068	
			267 F	264·39	39·2	1·83 ± 0·093	
			286	273·86	54·5	2·42 ± 0·079	
8	24–26	48–72	263	281·28	62·7	2·76 ± 0·076	5% = 0·37
			290	299·52	68·0	2·80 ± 0·076	1% = 0·51
			267 G	224·66	26·5	1·47 ± 0·046	0·1% = 0·70
			286 A	247·92	38·8	1·92 ± 0·158	
			291	258·73	46·5	2·16 ± 0·718	
			292	273·18	50·3	2·27 ± 0·088	

* See footnote to Table 3. The paired *t*-test was used only in Exp. 3.

The experiment just described showed that cystine, in fact, limits the b.v. of freshly prepared dried milk. Mitchell & Block(9) concluded, from microbiological assays, that cystine and methionine were limiting amino-acids in the proteins of fresh milk.

To confirm that unheated milk was deficient in cystine, tests were carried out with freeze-dried milk, as this is easier to include in a diet than milk in the liquid state.

A sample of bulk milk from the herd of the National Institute for Research in Dairying was freeze-dried, after separation, by Dr R. A. Kekwick of the Lister Institute, London. The b.v. and t.d. of this milk, alone or with cystine added at the same rate as in Exp. 1, were determined by the method of Mitchell(1, 2). Owing to difficulties in drying a sufficient quantity of milk a parallel test by the rat-growth technique(3) was not included.

Table 5. *Analysis of variance of the data obtained in all experiments*

Component	Degrees of freedom	Sum of squares	Mean square	Variance ratio
		Exp. 1. Biological value		
(i) Total	47	4571·87	—	—
(ii) Litters	2	194·64	97·32	ii/viii 8·57*, ii/vi 2·19 N.S.
(iii) Diets	3	3036·13	1012·04	iii/viii 89·06†, iii/v 38·76†
(iv) Periods	3	21·31	7·10	iv/viii 1·60 N.S., iv/vi 6·25‡
(v) Litters × diets	6	156·70	26·12	v/viii 2·30 N.S.
(vi) Litters × periods	6	266·36	44·39	vi/viii 3·91*
(vii) Rats within litters	9	692·21	76·91	vii/viii 6·77†
(viii) Error	18	204·52	11·36	—
		True digestibility		
(i) Total	47	1022·07	—	—
(ii) Litters	2	38·96	19·48	ii/viii 2·11 N.S., ii/vi 4·60 N.S.
(iii) Diets	3	636·22	212·07	iii/viii 22·93†, iii/v 83·51†
(iv) Periods	3	52·94	17·65	iv/viii 1·91 N.S., iv/vi 4·16 N.S.
(v) Litters × diets	6	15·23	2·54	v/viii 3·64‡
(vi) Litters × periods	6	25·44	4·24	vi/viii 2·18 N.S.
(vii) Rats within litters	9	86·73	9·64	vii/viii 1·04 N.S.
(viii) Error	18	166·55	9·25	—
		Exp. 4. Biological value		
(i) Total	47	4407·38	—	—
(ii) Litters	2	63·85	31·93	ii/viii 1·54 N.S., ii/vi 3·15 N.S.
(iii) Diets	3	3026·90	1008·97	iii/viii 486·6†, iii/v 128·0†
(iv) Periods	3	493·47	164·49	iv/viii 79·36†, iv/vi 16·24*
(v) Litters × diets	6	47·25	7·88	v/viii 2·63 N.S.
(vi) Litters × periods	6	60·76	10·13	vi/viii 2·05 N.S.
(vii) Rats within litters	9	341·97	38·00	vii/viii 1·83 N.S.
(viii) Error	18	373·18	20·73	—
		True digestibility		
(i) Total	47	534·02	—	—
(ii) Litters	2	7·15	3·58	ii/viii 2·30 N.S., ii/vi 1·38 N.S.
(iii) Diets	3	185·21	61·74	iii/viii 7·50*, iii/v 19·60*
(iv) Periods	3	65·44	21·81	iv/viii 2·65 N.S., iv/vi 8·42‡
(v) Litters × diets	6	18·87	3·15	v/viii 2·61 N.S.
(vi) Litters × periods	6	15·54	2·59	vi/viii 2·62 N.S.
(vii) Rats within litters	9	93·63	10·40	vii/viii 1·26 N.S.
(viii) Error	18	148·18	8·23	—
		Protein efficiency		
(i) Total	23	7·7619	—	—
(ii) Litters	5	0·5301	0·1060	ii/iv 1·77 N.S.
(iii) Diets	3	6·3335	2·1112	iii/iv 35·24†
(iv) Error	15	0·8983	0·0599	—
		Exp. 5. Biological value		
(i) Total	47	3393·98	—	—
(ii) Litters	2	22·62	11·31	ii/viii 1·73 N.S., ii/vi 1·34 N.S.
(iii) Diets	3	526·81	175·60	iii/viii 8·99†, iii/v 1·01 N.S.
(iv) Periods	3	742·56	247·52	iv/viii 12·67†, iv/vi 29·25†
(v) Litters × diets	6	1045·81	174·30	v/viii 8·92†
(vi) Litters × periods	6	50·78	8·46	vi/viii 2·31 N.S.
(vii) Rats within litters	9	653·73	72·64	vii/viii 3·72*
(viii) Error	18	351·67	19·54	—
		True digestibility		
(i) Total	47	469·54	—	—
(ii) Litters	2	69·69	34·85	ii/viii 9·93*, ii/vi 6·70‡
(iii) Diets	3	180·15	60·05	iii/viii 17·11†, iii/v 9·49‡
(iv) Periods	3	31·87	10·62	iv/viii 3·03 N.S., iv/vi 2·04 N.S.
(v) Litters × diets	6	38·00	6·33	v/viii 1·80 N.S.
(vi) Litters × periods	6	31·21	5·20	vi/viii 1·48 N.S.
(vii) Rats within litters	9	55·43	6·16	vii/viii 1·75 N.S.
(viii) Error	18	63·19	3·51	—

Table 5 (*continued*)

Component	Degrees of freedom	Sum of squares	Mean square	Variance ratio
		Protein efficiency		
(i) Total	23	2·5743	—	—
(ii) Litters	5	0·2331	0·0466	ii/iv 1·18 N.S.
(iii) Diets	3	1·7504	0·5835	iii/iv 14·81†
(iv) Error	15	0·5908	0·0394	—
		Exp. 6. Biological value		
(i) Total	47	5026·48	—	—
(ii) Litters	2	104·04	52·02	ii/viii 1·21 N.S., ii/vi 2·72 N.S.
(iii) Diets	3	1871·57	623·86	iii/viii 14·48†, iii/v 37·58†
(iv) Periods	3	1469·80	489·93	iv/viii 11·37†, iv/vi 25·60†
(v) Litters × diets	6	99·62	16·60	v/viii 2·60 N.S.
(vi) Litters × periods	6	114·81	19·14	vi/viii 2·25 N.S.
(vii) Rats within litters	9	591·09	65·67	vii/viii 1·52 N.S.
(viii) Error	18	775·55	43·08	—
		True digestibility		
(i) Total	47	518·29	—	—
(ii) Litters	2	84·63	42·32	ii/viii 9·20*, ii/vi 7·23‡
(iii) Diets	3	229·70	76·57	iii/viii 16·64†, iii/v 31·38†
(iv) Periods	3	0·99	0·33	iv/viii 13·94†, iv/vi 17·73*
(v) Litters × diets	6	14·64	2·44	v/viii 1·89 N.S.
(vi) Litters × periods	6	35·07	5·85	vi/viii 1·27 N.S.
(vii) Rats within litters	9	70·47	7·83	vii/viii 1·70 N.S.
(viii) Error	18	82·79	4·60	—
		Protein efficiency		
(i) Total	23	4·0143	—	—
(ii) Litters	5	0·3033	0·0607	ii/iv 2·54 N.S.
(iii) Diets	3	3·3522	1·1174	iii/iv 46·73†
(iv) Error	15	0·3588	0·0239	—
		Exp. 7. Biological value		
(i) Total	47	3125·91	—	—
(ii) Litters	2	88·99	44·50	ii/viii 4·24‡, ii/vi 3·82 N.S.
(iii) Diets	3	2016·17	672·06	iii/viii 64·01†, iii/v 15·83*
(iv) Periods	3	308·90	102·97	iv/viii 9·81†, iv/vi 8·83‡
(v) Litters × diets	6	254·78	42·46	v/viii 4·04*
(vi) Litters × periods	6	69·96	11·66	vi/viii 1·11 N.S.
(vii) Rats within litters	9	198·16	22·02	vii/viii 2·09 N.S.
(viii) Error	18	188·95	10·50	—
		True digestibility		
(i) Total	47	804·19	—	—
(ii) Litters	2	48·33	24·17	ii/viii 4·51‡, ii/vi 6·89‡
(iii) Diets	3	473·70	157·90	iii/viii 29·45†, iii/v 30·37†
(iv) Periods	3	5·74	1·91	iv/viii 2·81 N.S., iv/vi 1·84 N.S.
(v) Litters × diets	6	31·22	5·20	v/viii 1·03 N.S.
(vi) Litters × periods	6	21·04	3·51	vi/viii 1·53 N.S.
(vii) Rats within litters	9	127·66	14·18	vii/viii 2·65 N.S.
(viii) Error	18	96·50	5·36	—
		Protein efficiency		
(i) Total	35	4·1591	—	—
(ii) Litters	5	0·1331	0·0266	ii/iv 0·89 N.S.
(iii) Diets	5	3·2858	0·6572	iii/iv 22·20†
(iv) Error	25	0·7402	0·0296	—
		Exp. 8. Protein efficiency		
(i) Total	35	10·6341	—	—
(ii) Litters	5	0·7135	0·1427	ii/iv 1·60 N.S.
(iii) Diets	5	7·6936	1·5387	iii/iv 17·28†
(iv) Error	25	2·2270	0·0891	—

* = significant at 1 %. † = significant at 0·1 %.
‡ = significant at 5 %. N.S. = not significant.

The results of this experiment are shown in Table 3. With the addition of cystine, the b.v. increased from 90·1 to 97·3, the corresponding increase in t.d. was from 92·5 to 94·5; both increases were statistically significant.

These results confirm the view that cystine is a limiting factor in milk proteins, but suggest that this amino-acid is not affected by moderate heat treatment.

Comparison of the freshly prepared low- and medium-moisture control powders (*Exp.* 3)

A comparison was carried out, by the rat-growth method(3), of the freshly prepared samples of L and M powders in order to check that no marked differences existed between the nutritive values of their proteins (cf. p. 341). The results of this experiment are shown in Table 4. The values of 2·78 and 2·93 obtained for the p.e. of the L and M milks respectively are very similar and statistically indistinguishable. It may, therefore, be concluded that, as regards the quality of their proteins, no differences existed initially between the L, M and H powders.

Effect of storage at 37° C. *on the high-moisture powder* (*Exp.* 4)

The following H milks stored at 37° C. were selected, and the nutritive value of their proteins determined by both the balance-sheet(1, 2) and the rat-growth(3) methods: (1) Stored 8 days, air-packed; the flavour of this powder had seriously deteriorated but it was normal in other respects except for 'caking' due to crystallization of the lactose. (2) and (3) Stored 28 days, air-packed and gas-packed respectively; both powders showed marked loss of solubility at 20° C. and a slight loss at 50° C.; all oxygen was absorbed in the air-packed sample, i.e. there was maximum difference between air- and gas-pack. (4) Stored 85 days in air with resulting maximum development of insolubility.

A control sample was not included in this test. It was only possible to test four powders simultaneously by Mitchell's method(1, 2) and, on balance, it was considered that the four milks selected would yield the maximum information about the effects of storage.

The results of this experiment will be found in Tables 3, 4 and 5. The air-packed sample stored for 8 days had a b.v. of 86·9. This figure is somewhat lower than that obtained for the L control powder in Exp. 1, but it is higher than the values for the M control powder in Exps. 5 and 6, and almost the same as in Exp. 7. It may therefore be concluded that no deterioration in the b.v. had occurred after this period of storage. Storage of air-packed samples for 28 and 85 days resulted in a progressive and statistically significant lowering of the b.v. of the milk proteins to 78·8 and 65·9 respectively. The value of 83·3 obtained for the 28-day gas-packed sample was statistically superior to that found for the corresponding air-packed sample; it was lower, but not significantly so, than the figure found for the 8-day air-packed powder. It thus becomes evident that the changes on storage in the milk proteins are retarded under anaerobic conditions. The t.d. of the sample stored for 85 days was, statistically, markedly inferior to that of the other three milks, among which the small differences were not statistically significant.

There was a definite parallelism between the results obtained for the p.e. (Table 4) and those for the b.v. (Table 3), but the differences were less marked by the former method. This was particularly noticeable with the 28-day air- and gas-packed samples where the difference was not statistically significant.

Effect of moisture content and of temperature of storage on deterioration in samples of powder after 6 months' storage (Exp. 5)

After 6 months' storage at 37° C. M powder was showing signs of mild deterioration as judged by chemical tests, while H powder stored at 28·5° C. was showing more marked changes. Tests were therefore carried out on M control powder, on air-packed M powder stored at 37° C. for 6 months, and on H powder stored at 28·5° C. for 6 months, air- and gas-packed. This milk was tested in order to confirm the difference observed in Exp. 4 between air- and gas-packed samples. The tests were carried out by the nitrogen balance (1, 2) and the growth (3) methods.

The results are given in Tables 3–5. There was no decrease in the b.v. of M powder stored for 6 months at 37° C., but the t.d. showed a statistically significant decrease from 92·4 to 89·4. The air- and gas-packed samples of H powder stored for 6 months at 28·5° C. showed a significant decrease in both the b.v. and the t.d. The drop in b.v. was again more marked for the air- than for the gas-packed sample.

The results of the growth test again roughly confirmed those obtained by the balance sheet method but the differences lacked the same degree of significance.

Effect of the addition of 2·5% lysine to the deteriorated high-moisture powder and to control powder (Exp. 6)

In chemical tests Van Slyke estimations and formol titration indicated a change in the deteriorated milk powders whereby the free ϵ-amino-group of part of the lysine in the milk became blocked by a reaction, probably with carbohydrate (Part III). It seemed likely that lysine altered in this way would not be fully available to the rat, and that this change might be largely responsible for the lowered nutritive value of the milk. The two chemical methods did not indicate the same loss of amino-groups, the Van Slyke method showing a drop of some 60% during the storage of H powder at 37° C. for 60 days, the formol titration, of very much less (Part III). The results of the microbiological tests (Part V) were not yet available. In order to ensure full replacement of any lysine inactivated during storage, pure lysine was added to a control and to the deteriorated sample of milk at the rate of 2·5%, i.e. at the approximate original concentration ((10, 11, 12), Part V).

L-Lysine was added as the monohydrochloride dihydrate to M control powder and to a gas-packed sample of H powder, stored at 37° C. for 60 days. These mixtures were then analysed for N and made into diets to supply approximately 8% protein (cf. p. 341). Tests were carried out by both the balance-sheet (1, 2) and the rat-growth (3) techniques. The results are given in Tables 3–5.

It will be seen that both the b.v. and the p.e. of the deteriorated milk were markedly improved by the addition of 2·5% lysine. In neither case were the values obtained as high as those for the control milk; this difference was statistically significant for the p.e. but not for the b.v. The t.d. of the deteriorated powder was markedly lower than that of the control powder; the addition of the lysine, even if allowance is made for its complete digestibility, caused a statistically significant increase in the t.d., but the higher value obtained was still statistically inferior to that for the control powder.

The lowering of both the b.v. and the p.e. of the control powder by the addition of lysine is, at first sight, somewhat surprising. It is known that lysine is in no way a limiting

factor in milk(13) and this amino-acid, together with valine, is believed to be chiefly responsible for the supplementary relationship between the proteins of milk and wheat(14), the latter food being deficient in these amino-acids. Riesen, Schweigert & Elvehjem(15) noted an improvement in the efficiency of nitrogen utilization by rats when 0·22 L-cystine or 0·4% DL-methionine were added to casein, but little or no improvement when the two amino-acids were added together. They suggested that this finding might be due to an imbalance of amino-acids when the two acids were added simultaneously. Somewhat similar observations were made in human experiments by Murlin, Edwards, Hawley & Clark(16) who studied the effects of added amino-acids on the biological value of egg- and soya bean-proteins. It is possible that such an effect may account for our findings. On the other hand, as milk contains adequate amounts of lysine, it may be that the rat simply does not utilize the amino-acid added to the control powder, and that this nitrogen is excreted in the urine. The b.v. and the p.e. values for the control powder to which lysine had been added were therefore recalculated on the assumption that the lysine nitrogen was not utilized. For this purpose the lysine nitrogen was subtracted from the urinary nitrogen in the measurements by the Mitchell method(1, 2) or from the nitrogen intake in measurements by the growth technique(3). The values of 84·3 and 2·85, respectively, so obtained are very close to the corresponding figures of 84·5 and 2·81 obtained for the control milk alone.

The results of the microbiological assays (Part V) were available when this experiment was completed. They indicated that, in H powder stored in air-pack at 37° C. for 95 days, some 40% of the lysine originally present resisted enzymic hydrolysis. This suggested that in the experiment just described, an excess of lysine had been added even to the deteriorated powder and that this excess may have prevented the b.v. and the p.e. of the supplemented deteriorated powder from reaching those of the control powder. The alternative explanation that, besides lysine, other essential amino-acids had been affected was by no means ruled out but, before this possibility was investigated, further tests were done with smaller additions of lysine.

Effect of the addition of 1·25% *lysine to the deteriorated high-moisture powder, and of lysine at several levels to the control powder* (*Exp.* 7)

As mentioned above, microbiological tests indicated that rather less than 50% of the lysine originally present in milk had, after storage, become resistant to enzymic hydrolysis. It was decided, therefore, to add 1·25% lysine to a deteriorated powder. If lysine was the only amino-acid affected, its addition at this rate should cause full restoration to the initial b.v. At the same time it was considered that a study of the addition of graded amounts of lysine to the control powder would be profitable. It was not practicable to test more than six samples of powder and the following were chosen: (1) M control; (2) M control with 1·25% lysine; (3) M control with 2·5% lysine; (4) M control with 5% lysine; (5) H powder, gas-packed ,stored for 60 days at 37° C.; (6) H powder, gas-packed, stored for 60 days at 37° C., with 1·25% lysine. In making the diets allowance was made for the nitrogen supplied by lysine (cf. Exp. 6). All samples were tested by the growth method(3); in addition, samples (1), (2), (5) and (6) were tested by the balance-sheet method(1, 2). The results of this experiment are shown in Tables 3–5.

The addition of 1·25% lysine to the deteriorated powder raised its b.v. almost to the level of the control powder, the difference between these two being without statistical

significance. The addition of lysine to the control powder again caused a significant lowering of the b.v. If it is assumed that this added lysine was not required by the rats, a recalculation of the b.v. gives a value of 88·0, almost identical with the value of 87·6 obtained for the M control powder. This finding lends strong support to the belief, expressed above, that the lowered b.v. of high-quality powder observed after the addition of lysine is due to the superfluity of the latter. The matter is being put to a more direct test.

The t.d. of the deteriorated powder was found to have fallen, as in previous experiments; the addition of 1·25% lysine caused an increase which was not statistically significant.

The figures for the p.e. again followed the same trend as those obtained for the b.v. but, as in the earlier experiments, the differences were less marked. The addition of 1·25% lysine to the deteriorated powder caused a marked increase, but the value obtained was still inferior to that found for M control powder. In this test the addition of 1·25 and 2·5% lysine to M control powder had little or no effect on its p.e., but 5% lysine caused a significant lowering. If it is again assumed that this lysine was in excess of the animals' requirements, and allowance is made for this, the value of 2·35 becomes 2·73, almost identical with that of 2·71 obtained for the M control powder; a similar calculation gives a value of 2·81 for the powders with the addition of 1·25 and 2·5% lysine.

Effect of the addition of lysine, histidine and arginine to the deteriorated high-moisture powder (*Exp.* 8)

It was found in Exp. 7 that, although the addition of 1·25% lysine to the deteriorated powder largely restored the b.v. to its initial value, the improvement in the t.d. and p.e. was less satisfactory. The results of the microbiological assay (Part V) suggested that histidine, arginine, and possibly methionine might also have been affected, although much less than lysine. Supplies of the powders originally prepared for these tests were running low and it was not possible to carry out tests by both the balance-sheet (1, 2) and the growth (3) techniques; in addition only a sample of H powder which had been stored at 37° C. for 60 days in the presence of a relatively large supply of air (cf. Part III, p. 309) was available. It had been found in Exps. 4 and 5 that deterioration was more marked in air-pack than in gas-pack. A preliminary test was carried out using only the less laborious growth method (3) to study the effects of the addition of histidine and arginine as well as lysine to the deteriorated powder. L-Arginine and L-histidine were added to the powders as the monohydrochlorides at the rate of 0·5%, that is about 30% of the amount of these acids originally present, as the microbiological tests indicated a possible loss of this order (Part V). The following samples of powder were tested: (1) M control; (2) M control with 0·5% histidine and 0·5% arginine; (3) H powder air-packed, stored for 60 days at 37° C.; (4) H powder, air-packed, stored for 60 days at 37° C., with 1·25% lysine; (5) H powder, air-packed, stored for 60 days at 37° C., with 1·25% lysine and 0·5% histidine; (6) H powder, air-packed, stored for 60 days at 37° C., with 1·25% lysine, 0·5% histidine and 0·5% arginine. The results of this experiment are shown in Tables 4 and 5.

It will be seen that the addition of histidine and of arginine to the deteriorated powder supplemented with lysine caused an improvement in the p.e. which, though suggestive, just lacked conventional statistical significance. The value obtained with these three

amino-acids still fell short of that observed for the M control powder. Nevertheless, the result suggested that this further addition of histidine and arginine to the deteriorated powder supplemented with lysine had had a beneficial effect. It should be noted that the addition of histidine and arginine did not depress the p.e. of the control powder, although the total addition of these acids amounted to 1%. This is in line with the finding that the addition of 1·25% lysine did not depress the p.e. of the control milk although it depressed its b.v. (Exp. 7). It is hoped to repeat this experiment at a later date using Mitchell's method (1, 2). It must be borne in mind that the deteriorated sample used in this experiment was packed in air, and not in nitrogen as in Exp. 7, and that the amount of oxygen which had been absorbed by this powder was some three times greater than with the air-packed samples tested in Exps. 4 and 5.

DISCUSSION

The experiments described above confirm our earlier findings (6, 7) that the proteins of dried skim milk undergo marked deterioration in nutritive value on storage. They show that the moisture content of the powder has a determining influence in the development of this fault. The lowering of the nutritive value of milk proteins was chiefly due to changes in the availability of lysine. In fact, if the probable loss of this acid, as judged by amino-nitrogen measurements (Part III) and by microbiological assay after enzymic hydrolysis (Part V), was made good, the b.v. of a gas-stored deteriorated powder could be restored to its original value (Exp. 7). On the other hand, this addition of lysine only partly restored the p.e. of the powder. It stands to reason that the latter value will be influenced, to some extent at any rate, by the lowered digestibility of the proteins of deteriorated powder and that, in judging the efficiency of its proteins to promote growth, only the value of that part which is available should be considered. Table 6 gives such

Table 6. *Protein efficiencies of the deteriorated milks stored at* 37° C. *for* 60 *days, supplemented with amino-acids, recalculated from values given in Table* 4 *by making allowance for the lowered digestibility of the milk proteins as indicated by findings in tests by the balance-sheet method quoted in Table* 3.

Exp. no.	Type of packing	Supplement	Value found	'Correeted' value*	Value for M eontrol milk
6	Gas	2·5% lysine	2·55	2·74	2·81
7		1·25% lysine	2·42	2·60	2·71
8	Air	1·25% lysine	1·92	2·07	2·76
8		1·25% lysine + 0·5% histidine	2·16	2·32	2·76
8		1·25% lysine + 0·5% histidine + 0·5% arginine	2·27	2·44	2·76

* In Exps. 6 and 7 the t.d. of the deteriorated milk was only 93% of that of the original milk as measured by the parallel Mitchell (1, 2) test, the same figure was assumed for Exp. 8 where no Mitchell tests were carried out.

'corrected' values, based on t.d. found by the Mitchell technique in Exps. 6 and 7. These calculations show that for Exps. 6 and 7, where a gas-packed sample of powder was used, the p.e. was largely restored to the value obtained for M control powder; with the air-packed sample used in Exp. 8 the gap between the 'corrected' and the 'control' figures still remained appreciable. This suggests that, for air-stored powder at least, a further amino-acid or acids, in addition to lysine, is involved, and is in line with the findings of Exps. 4 and 5 where the b.v. of milk deteriorated more markedly on storage in an air-pack than when gas-packing was used.

It will have been noticed that on several occasions the addition of an amino-acid to a milk powder increased not only its b.v. but also its t.d. It is not clear how this second effect is brought about and why the absorbability of a protein should be altered by the addition to it of an amino-acid. To a certain extent the change is only apparent as the added amino-acid is, no doubt, 100% digestible, and this influences the value for the t.d. of the mixture. By allowing for this influence, it can be calculated, for example, that in Exp. 6 the t.d. of the deteriorated powder to which 2·5% lysine had been added should be 88·1 and not 89·0. This corrected value is no longer significantly different from that (86·0) for the unsupplemented powder. In this case, therefore, the improvement was most probably fortuitous. Confirmation of this is found in Exp. 7 where the addition of 1·25% lysine to the deteriorated powder increased the t.d. from 85·1 to 87·0; this difference was, however, below the conventional level of significance and, when allowance is made for the complete absorption of the lysine nitrogen, the corrected figure of 86·3 is, of course, statistically indistinguishable from the original value. The increase in t.d. of freshly prepared powders after the addition of cystine in Exps. 1 and 2 cannot, however, be explained in the same way, as the amount of cystine nitrogen was relatively very small and allowance for it does not alter the statistical significance of the difference. It is of interest that the t.d. of the deteriorated sample was not affected by the addition of cystine, whereas lysine caused an apparent improvement only with the deteriorated powders.

The results of Exp. 8 suggest that, in addition to lysine, histidine, alone or in combination with arginine, might completely restore the nutritive value of the proteins of deteriorated gas-packed powder. As shown below, the necessity of adding arginine is doubtful on theoretical grounds, and in effect it caused only an insignificant improvement when added in combination with histidine and lysine. It has been shown by Scull & Rose (17) that the rat is able to synthesize arginine but the rate of synthesis is not sufficient to maintain normal growth and the presence of dietary arginine is essential (18). Table 7

Table 7. *Percentages of essential amino-acids in the experimental diets (containing 23% dried milk) calculated on the basis of the microbiological data (Part V) for undeteriorated control milk, compared with Rose's (22) figures for the quantities needed to satisfy minimum requirements for normal growth of rats*

Amino-acid	Calculated amount in diet (%)	Rose's minimum requirements (%)
Arginine	0·32	0·2
Histidine	0·32	0·4
Isoleucine	0·48	0·5
Leucine	1·53	0·9
Lysine	0·61	1·0
Methionine	0·22	0·6
Phenylalanine	0·37	0·7
Threonine	0·59	0·6
Tryptophan	0·10	0·2
Valine	0·56	0·7
Cystine	0·05	—

compares the approximate amounts of the essential amino-acids, likely to be available in the control milk diets fed to rats in the present experiments, with Rose's (18) figures for the minimum amounts needed for normal growth when non-essential acids are included in the diet. It will be seen from this table that only arginine and leucine were supplied in excess of the animals' minimum requirements for individual amino-acids. It should be noted, however, that at the 8% level of protein intake at which our tests were done, the

growth rate of rats is subnormal and that their requirements would be below the minimum values quoted in Table 7.

Even a 30% loss of arginine still leaves this acid at approximately the minimum figure postulated by Rose (18). It therefore seems unlikely that addition of arginine would appreciably enhance the nutritive value of the proteins of the deteriorated powder.

Briefly stated, the biological tests give the following picture of the changes in milk powder of high moisture content brought about by storage. A loss or inactivation of lysine accounts for the bulk of the depression of the b.v.; this may be accompanied or followed by a slight loss of histidine. Inactivation of lysine is probably dependent on the atmosphere in the container only in so far as the reaction proceeds more rapidly after the crystallization of lactose which, in these experiments, occurred slightly earlier in the air- than in the gas-packed powders (Part III, p. 324). The loss of histidine may require, or be increased by, the presence of oxygen, but further work is necessary to confirm the loss of this acid and to ascertain the influence of oxygen on the process. The microbiological tests (Part V) indicate the possibility of a slight loss of methionine during storage, but tests with this amino-acid have not yet been made with rats. The overriding influence of the moisture content of the powder on the inactivation of lysine has already been stressed (p. 338); it is not possible to say at present what is the influence of moisture content on the inactivation of acids other than lysine.

It is not yet known whether milks of low moisture content are also liable to these oxidative changes. None of the low-moisture milks were tested biologically because chemical and physical tests (Part III) indicated little or no change.

It has already been mentioned that good parallelism was observed between the results for the b.v. of the milk powder obtained by the balance-sheet method (1, 2) and the p.e. measured in *ad lib.* feeding tests by the growth technique of Osborne, Mendel & Ferry (3).

The correlation between the results obtained by the two methods is shown graphically in Fig. 1. The agreement is excellent as indicated by the high correlation coefficient of 0·89, and there is no doubt that, in our experience at any rate, the simple growth technique has proved most valuable in assessing the quality of a protein. It is rather less sensitive to small differences than the balance-sheet method (1, 2), but is specially useful in pilot experiments.

Our findings are in agreement with results obtained by Carlson, Hafner & Hayward (14) who applied both these methods. When they used equalized feeding for the p.e. measurements, agreement with Mitchell's method (1, 2) was rather less satisfactory. Barnes, Maack, Knights & Burr (19) found that, for good quality proteins with a p.e. of about 2·0, *ad lib.* feeding tests gave, at a 10% level of protein intake, more uniform results than equalized feeding tests; lower results were obtained by the latter technique. They criticize the method strongly because it does not take into account the digestibility of the proteins tested. Mitchell (20) is of the opinion that the balance-sheet method (1, 2) is more accurate and detects smaller differences than the growth method (3), and we would be inclined to support this view. Mitchell (20) shows further that *ad lib.* feeding in the growth method may be expected, on account of its large experimental error, to exaggerate quality differences among proteins when these are large and to obscure them when they are small. He found that the food intake was usually greater for the better quality proteins; this was to some extent confirmed by the present experiments. Reference to Table 4 shows that the diet intake was, as a rule, greater with milks of higher p.e., but on three

occasions (diets 263A and 267D, Exp. 5; diets 263 and 283, Exp. 6; diets 263 and 285, Exp. 7) the difference was either negligible or in the other direction, though in all three cases the differences in p.e. were statistically significant.

Graves (21, 22) has strongly criticized the conclusions of Chick (23) based on tests by the growth method (3), contending that her results could have been predicted from the diet intakes of the rats. Chick's results were subsequently confirmed in this laboratory (24) by the balance-sheet method (1, 2). Graves (22) also criticized, on the same score, certain conclusions reached by Henry & Kon (25). This criticism is vitiated by the unwarranted

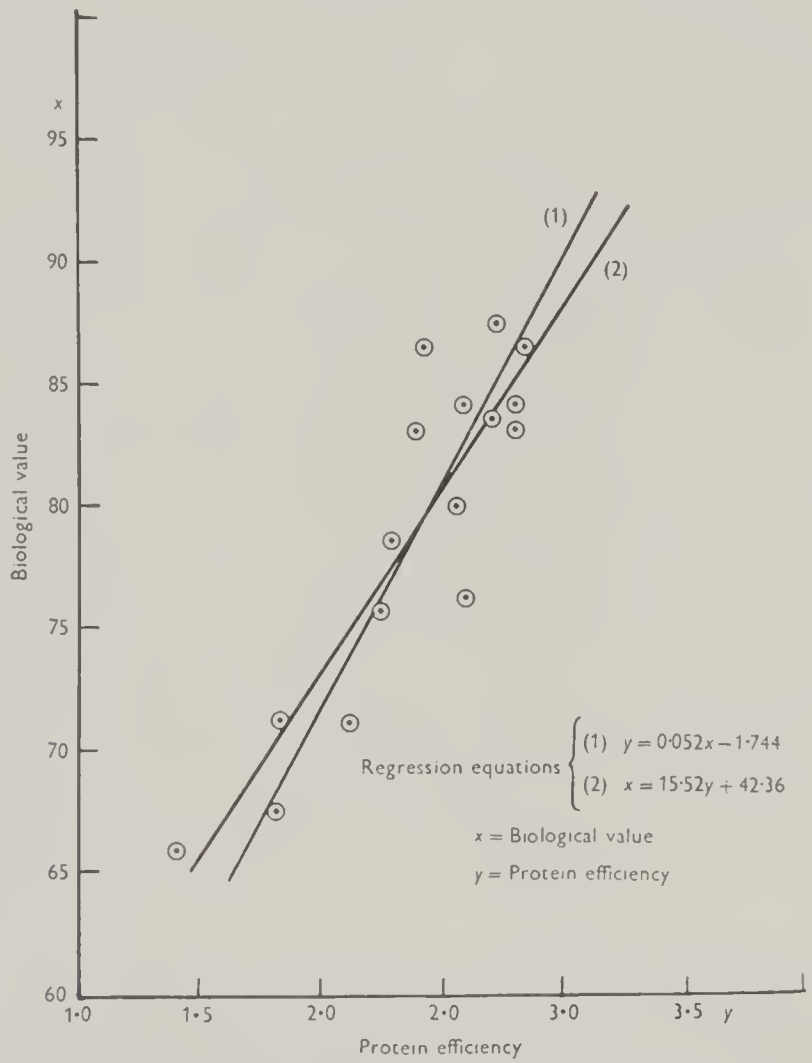

Fig. 1. The relationship between the biological value and the protein efficiency of milk proteins.

rejection by Graves of some of their data, apparently occasioned by his incorrect interpretation of the meaning of a passage in their paper. The present findings make his contention, in any case, untenable.

Summary

1. To investigate the causes and nature of the deterioration in nutritive value on storage of the proteins of dried skim milk (6, 7), biological tests with rats were carried out with a number of powders of different moisture content stored under different conditions. The experiments were done by the balance-sheet method of Mitchell (1, 2) and by the growth method of Osborne, Mendel & Ferry (3). Suitable samples of powder were chosen for this purpose on the basis of the chemical tests described in Part III of this paper.

2. The biological value of the proteins of dried skim milk of high moisture content (7·6 %) decreased progressively during storage in air at 37° C.. Storage for 8 days produced no perceptible change (b.v. 86·9) but the value was 78·8 after 28 days and 65·9 after 85 days.

3. Under these conditions the true digestibility of the milk proteins did not alter after storage for 1 month, but a statistically significant decrease of 5–6 % occurred by the end of 2 months; little change was observed after longer periods of storage.

4. At the lower storage temperature of 28·5° C. the change in high-moisture samples was about six times slower than at 37° C., the biological value and true digestibility of the proteins of a sample stored for 6 months being comparable with those of the sample stored for 1 month at 37° C.

5. Powder with a lower moisture content (5 %) proved much more stable; the biological value of its proteins was unchanged after storage for 6 months at 37° C. though the true digestibility of the proteins decreased significantly by 4 %.

6. Dried skim milks with the still lower content of 3 % moisture were not examined, after storage, in these biological tests, as chemical tests showed that they suffered little deterioration under any conditions of storage.

7. The decrease in the biological value of the proteins was slightly more marked in air-packed than in gas-packed samples of milk. The true digestibility of the proteins was not affected by the type of packing.

8. Although differences were, in general, less marked, the values for the protein efficiency of the milks followed the same trend as those for the biological value of the proteins. The agreement between the two methods was good as shown by the high correlation coefficient of 0·89 for all results.

9. The addition of 1·25 % lysine to a sample of high-moisture powder stored for 60 days at 37° C. in nitrogen, increased the biological value of the proteins approximately to the figure obtained for a control sample. An increase in the true digestibility of the proteins and in the protein efficiency of the milk was also observed but the values obtained fell short of those for the control milk.

10. The addition of histidine as well as lysine caused a further slight improvement in the protein efficiency of a sample of deteriorated milk which had been stored for 60 days at 37° C. in air, but arginine was without effect.

11. The addition of lysine to the control powder led to a significant lowering in both the biological value of the proteins and the protein efficiency. This was most probably not because the added lysine was harmful but because, given in excess, it was not utilized.

12. The addition of cystine to fresh samples of spray-dried and of freeze-dried skim milk significantly improved both the biological value and the true digestibility of the proteins of these milks.

13. The results thus indicate that a high moisture content in dried skim milk is the most important factor in the deterioration of the milk proteins. Most of the decrease in the biological value of the proteins of such milks is accounted for by the loss or inactivation of lysine; it is probable that some loss of histidine also occurs.

We are indebted to Prof. A. C. Chibnall, F.R.S., to Dr W. E. Gaunt and to Dr S. J. Rowland for helpful advice. Our thanks are due to Dr R. A. Kekwick for the preparation of the freeze-dried milk; to Miss M. V. Chapman for help with the care of the experimental animals and to Miss J. Wagnell for help with some of the nitrogen analyses.

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PART V. MICROBIOLOGICAL ASSAY OF 'ESSENTIAL' AMINO-ACIDS

By KATHLEEN M. HENRY, S. K. KON, C. H. LEA and J. C. D. WHITE

(With 1 Figure)

For the estimation of the ten amino-acids regarded as essential for the rat and of cystine, a control milk powder and six samples of deteriorated powder were selected. The deteriorated powders were all of high moisture content (H powder, cf. Part II), and had been stored at 37° C. for 10 days (air), 30 days (air), 30 days (gas) and 95 days (air), or at 28·5° C. for 176 days (air) and 176 days (gas) respectively. Samples were chosen to correspond as closely as possible to those which had been tested biologically as described in Part IV.

Method

The analyses were carried out for the authors by Dr E. C. Barton-Wright who used the methods of microbiological assay described by him (1). Since preliminary tests for several individual amino-acids carried out after hydrolysis of the powder showed little or no loss even in badly deteriorated samples, it was decided to apply enzymic hydrolysis with pancreatin, and to use acid hydrolysis of the control and of one badly deteriorated sample for purpose of comparison only.

There are several disadvantages in the use of the enzymic method, the most serious being cessation of hydrolysis before the protein is completely broken down to amino-acids. In the present work recovery of the eleven acids after treatment of the powders with pancreatin was only of the order of 55–60% of the recovery obtained after hydrolysis with acid (Table 1). In order to minimize errors introduced by inequalities in the degree of hydrolysis undergone by the various samples (the control, for example, showed an appreciably higher total recovery of acids than any of the stored samples) the results for individual acids have been recalculated in Table 2 as a percentage of the total amount of the eleven acids recovered. This device should serve to expose selective loss of a particular acid or acids. In the event of such loss the percentage of the remaining acids in the mixture would, of course, rise slightly in compensation. Since most of the chemical criteria measured (Part III) indicate that storage for 176 days at 28·5° C. is roughly equivalent to 30 (or perhaps slightly more) days at 37° C.—a result with which the nutritional data (Part IV) are also in agreement—the 28·5° C. samples have been included with those stored at 37° C. at an appropriate place in the tables. For the preparation of Fig. 1, the 30 days 37° C. and 176 days 28·5° C. samples stored in air and in nitrogen have been treated as replicates, and the four values averaged.

RESULTS

The error inherent in the estimation of 'essential' amino-acids in proteins by the microbiological method has been given as ±15% (2), although individual workers frequently claim a degree of reproducibility considerably higher than this. In the present work the carrying out of the assays directly on milk powder, and the special difficulties introduced by the necessity for employing enzymic hydrolysis militated against obtaining maximum accuracy. Within the limitations of the methods used, it can be seen that deterioration of the powder during storage caused no loss of threonine, leucine, valine, isoleucine, phenylalanine, tryptophan or cystine, whether hydrolysis was by acid or by enzyme. Histidine showed a slight loss both by acid and by enzyme, and arginine and methionine a slight loss by enzyme but not by acid (Table 2, Fig. 1). These changes, however, were small in relation to the possible errors in the determinations, and must be considered as of doubtful significance. Only with lysine was there reasonably strong evidence of apparent loss during storage, this being considerably greater when measured after hydrolysis with enzyme than when acid hydrolysis was used. This observation is in agreement with results recently reported by Eldred & Rodney (3) for heat-damaged casein, in which the amount of free lysine released by enzyme was considerably reduced, although the amount liberated by acid hydrolysis was only very slightly reduced. In both forms of deterioration there is the

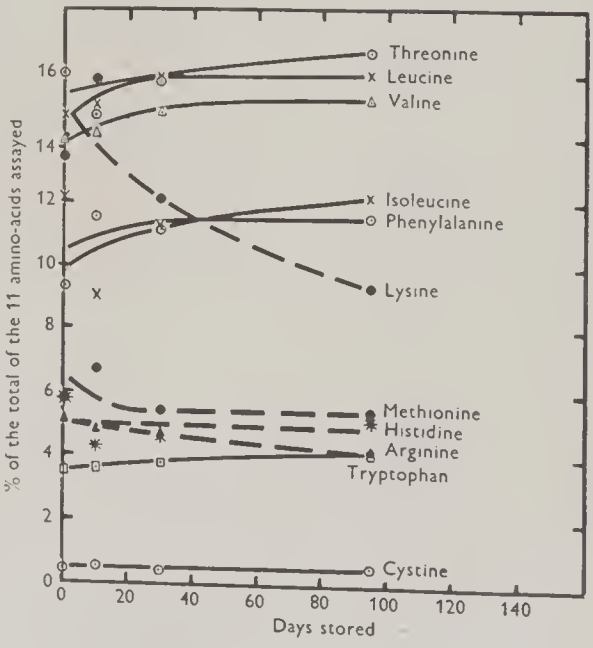

Fig. 1. Changes in content of 'essential' amino-acids during the storage of milk powder at 37° C. (enzymic hydrolysis).

Table 1. *Results of microbiological measurements of amino-acids in fresh and stored milk powders after hydrolysis with acid and with enzyme*

Days stored	Temp. (° C.)	Atmosphere	Method of hydrolysis	Amount of each acid found, as percentage of the dry powder: Lysine	Histidine	Arginine	Tryptophan	Phenylalanine	Leucine	Isoleucine	Valine	Threonine	Methionine	Cystine	Total
0	—	—	Acid*	2·65	1·40	1·37	0·45	1·60	3·02	2·10	2·43	2·58	0·94	0·22	18·76
95	37	Air	Acid*	2·04	1·10	1·34	0·40	1·45	2·95	1·90	2·22	2·54	0·86	0·20	17·00
0	—	—	Enzyme	1·70	0·74	0·65	0·44	1·18	1·86	1·53	1·76	2·03	0·73	0·05	12·67
10	37	Air	Enzyme	1·75	0·48	0·53	0·40	1·28	1·66	1·00	1·56	1·63	0·74	0·05	11·08
30	37	Air	Enzyme	1·50	0·48	0·53	0·43	1·30	1·66	1·03	1·57	1·81	0·70	0·05	11·06
30	37	N_2	Enzyme	1·30	0·58	0·52	0·46	1·28	1·94	1·40	1·76	1·66	0·51	0·05	11·46
176	28·5	Air	Enzyme	1·33	0·53	0·52	0·45	1·30	1·86	1·42	1·76	1·78	0·70	0·05	11·70
176	28·5	N_2	Enzyme	1·29	0·48	0·53	0·36	1·10	1·66	1·20	1·57	1·83	0·51	0·05	10·58
95	37	Air	Enzyme	0·96	0·53	0·42	0·42	1·19	1·66	1·27	1·57	1·73	0·56	0·05	10·36

* Tryptophan was measured after hydrolysis with barium hydroxide.

Table 2. *Content of individual amino-acids measured microbiologically in milk powders, expressed as a percentage of the total of the eleven acids tested*

Days stored	Temp. (° C.)	Atmosphere	Method of hydrolysis	Lysine	Histidine	Arginine	Tryptophan	Phenylalanine	Leucine	Isoleucine	Valine	Threonine	Methionine	Cystine
0	—	—	Acid*	14·1	7·5	7·3	2·4	8·5	16·1	11·2	13·0	13·7	5·0	1·2
95	37	Air	Acid*	12·0	6·5	7·9	2·4	8·5	17·3	11·2	13·1	14·9	5·1	1·2
0	—	—	Enzyme	13·4	5·8	5·1	3·5	9·3	14·7	12·1	13·9	16·0	5·8	0·4
10	37	Air	Enzyme	15·8	4·3	4·8	3·6	11·5	15·0	9·0	14·1	14·7	6·7	0·5
30	37	Air	Enzyme	13·6	4·3	4·8	3·9	11·7	15·0	9·3	14·2	16·4	6·3	0·5
30	37	N_2	Enzyme	11·3	5·1	4·5	4·0	11·2	16·9	12·2	15·4	14·5	4·5	0·4
176	28·5	Air	Enzyme	11·4	4·5	4·4	3·9	11·1	15·9	12·1	15·0	15·2	6·0	0·4
176	28·5	N_2	Enzyme	12·2	4·5	5·0	3·4	10·4	15·7	11·3	14·8	17·3	4·8	0·5
Mean of 30 and 176 day values			Enzyme	12·1	4·6	4·7	3·8	11·1	15·9	11·2	14·8	15·8	5·4	0·4
95	37	Air	Enzyme	9·3	5·1	4·1	4·1	11·5	16·0	12·2	15·2	16·7	5·4	0·5

* Tryptophan was measured after hydrolysis with barium hydroxide.

suggestion of combination of the ε-amino-groups of the lysine residues in linkages more resistant to proteolytic enzymes than to acid.

Hodson & Krueger(4) have carried out microbiological tests of amino-acids after acid hydrolysis in freshly prepared dried skim milk and in samples which had been stored for 51 months at room temperature in air-tight cans or in cans with loose lids. They noted small losses of lysine, histidine, methionine and arginine in the samples stored in the loose-lidded cans.

SUMMARY

Microbiological tests of fresh and stored separated milk powders showed a definite apparent loss of lysine in deteriorated powder, the loss being greater when measured after enzymic, than after acid, hydrolysis. The maximum deficiency of lysine recorded was about 40% of the original content of this acid.

A slight loss of histidine was also probable, and of arginine and methionine possible, but the reproducibility of the methods was not sufficient to establish these with certainty.

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PART VI. GENERAL DISCUSSION

BY KATHLEEN M. HENRY, S. K. KON, C. H. LEA AND J. C. D. WHITE

(With 2 Figures)

Questions of physical and chemical changes, and of palatability, in the milk powders during storage, and those dealing with the work on the nutritive value of the proteins, and with the microbiological assay of amino-acids have already been discussed and summarized in the appropriate sections of Parts III, IV and V. There still remain to be considered, in relation to one another, the conclusions reached from the very different approaches to the problem which have been described in the foregoing parts.

The chemical data show that a reaction resulting in the destruction or 'blocking' of free amino-groups of the milk protein—which must consist largely of the ε-amino-groups of the lysine residues—occurs in powder of high moisture content during storage, particularly at high temperatures. Evidence is produced in support of the view that these changes originate from a reaction between the protein amino-groups and reducing sugar, very largely if not entirely, lactose. The data suggest, furthermore, that most of the objectionable physical and chemical changes in such powders, e.g. in palatability, colour and solubility, are connected with this reaction. Exclusion of atmospheric oxygen by packing in inert gas has little or no direct effect on the primary protein-sugar reaction, a small protective effect observed with nitrogen-packed powder being attributed to a slight retardation of crystallization of the lactose under these conditions. Subsequent degradation of the protein-sugar complex is influenced by the storage atmosphere, as is shown by the considerable absorption of oxygen by the air-packed material and by the more rapid production of carbon dioxide and development of 'off'-flavour under these

conditions. The protein-sugar reaction is profoundly influenced by the moisture conten of the powder, and has a high temperature coefficient. At low moisture contents th reaction is almost completely prevented.

In addition to the primary protein-sugar reaction and to secondary changes resultin from it, evidence has been obtained, by gas exchange and palatability tests, of oxidativ changes much less dependent on a high moisture content in the powder and possessin a much lower temperature coefficient. While the nature of these reactions has not bee elucidated, it is probable that they include autoxidation of the trace of residual fat presen in the powders.

Comparison of the chemical and biological data

The relation between the observed reduction in free amino-nitrogen and the loss o nutritive value of the proteins in the stored powders, as determined by the two biologica methods used, is shown in Fig. 1. In the upper portion of the figure the biological value

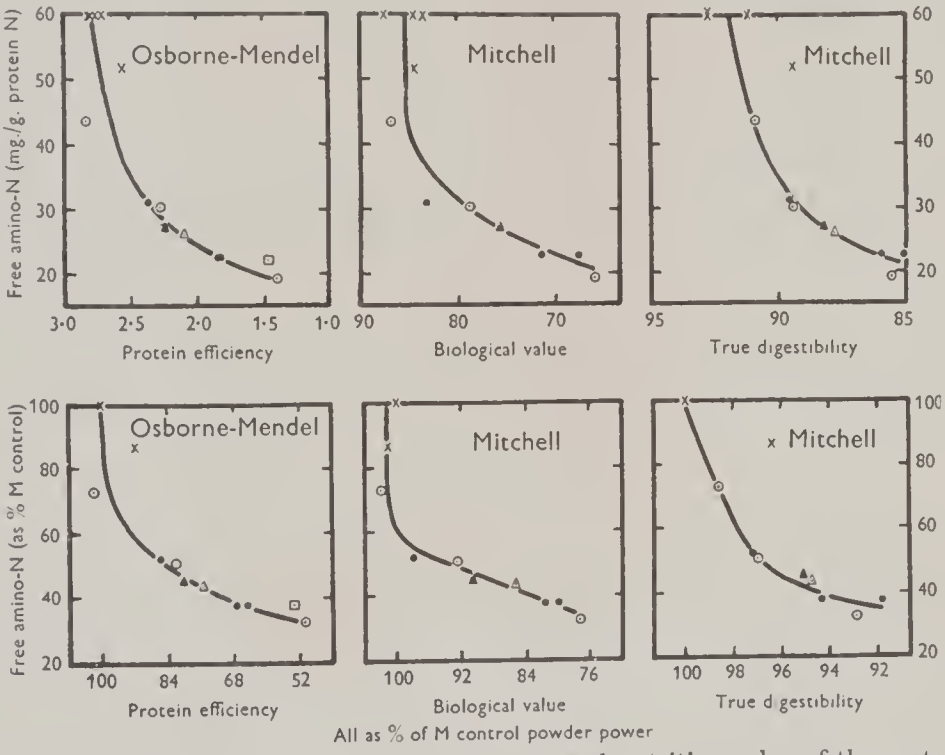

Fig. 1. The relationship between free amino-nitrogen content and nutritive value of the proteins. H powde 37° C. air = ⊙; H powder, 37° C. 'extra air' = ⊡; H powder, 28·5° C. air = △; H powder, 37° C nitrogen = ●; H powder, 28·5° C. nitrogen = ▲; M powder, control or 37° C. air = ×.

have been plotted as determined, the various data for M control powder obtained a different times with the different groups of rats being included separately. In the lowe part of the figure all the values obtained in one experiment with one group of rats hav been expressed as percentages of the value for the fresh, control powder in that experi ment. For the one series which did not include a control, an average value based on all th available determinations was used.

The experimental points, which are largely derived from H powder stored in nitroge or in a restricted supply of air at 37 or 28·5° C. for periods up to 6 months, lie on a reason ably smooth curve. This suggests that the blocking of free amino-groups of the protei leading to the inactivation of lysine was probably a major factor in producing the loss o nutritive value observed for these powders. The Osborne-Mendel and Mitchell data agre in showing little or no loss in nutritive value during the early stages of the protein-suga

reaction, but a comparatively rapid loss later. This may be due to either or both of the following reasons. (1) Milk protein is rich in lysine, and it might be expected that this amino-acid would not become a limiting factor in determining the biological value of the protein until an appreciable proportion of it had been inactivated. (2) In the early stages of the protein-sugar reaction, when neither protein nor carbohydrate has undergone serious secondary change, it may be that the link between the ε-amino-groups of the lysine residues and sugar can be more easily split by the digestive enzymes with regeneration and utilization of part or all of the combined lysine. At a later stage of the process the regeneration of lysine may be more difficult or impossible.

With a deteriorated milk powder, determination of the free amino-nitrogen content of the intact protein yields values for lysine lower than those given by microbiological assay of lysine after enzymic hydrolysis of the protein (Part V) and these in turn are lower than values obtained in the same way after acid hydrolysis. The original lysine content of the fresh powder is never reached, however, and a rough estimate of the figures for a 37° C., 60-day, gas-stored H powder might be given as of the order of 40, 70 and 90% recovery respectively. This change in the recovery of lysine according to method of treatment suggests that the rat may be able to utilize a proportion of the bound lysine. In this

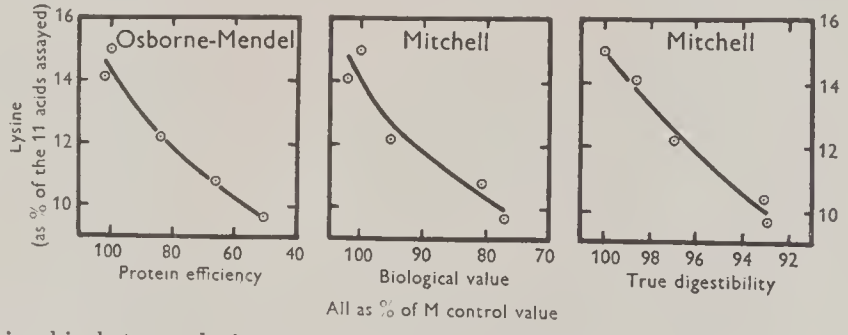

Fig. 2. The relationship between lysine content, as measured microbiologically after enzymic hydrolysis, and nutritive value of the proteins.

connexion it should be borne in mind that enzymic digestion prior to microbiological assay was continued for 5 days, and that Melnick, Oser & Weiss(1) have pointed out that for optimum utilization of food protein not only must all essential amino-acids be present, but they must also be liberated during digestion *in vivo* at rates permitting mutual supplementation. Omission of a single essential acid results in a poor utilization of the remainder which cannot be corrected for by injection of the missing acid some hours later(2). In the same way Henry & Kon(3) have demonstrated that supplementary relationships between two proteins only become evident when these are fed simultaneously and not when they are only available at different times.

Comparison of the results obtained in the rat-feeding experiments with those given by microbiological assay of lysine after enzymic hydrolysis of the deteriorated powders can only be made roughly, owing to the limitations of the microbiological data available. In Fig. 2, lysine values read off from Fig. 1, Part V, have been plotted against the nutritive value of the proteins as determined by the two biological methods. Only the biological results for H powder at 37° C. have been used, and data on air- and gas-stored powders have been averaged in order to bring them into line with the simplifications employed in deriving the lysine values. From Fig. 2 it can be seen that a much more direct relationship exists between loss of lysine, as determined by microbiological assay after enzymic

hydrolysis, and reduction in nutritive value, than between the latter and reduction in free amino-nitrogen (Fig. 1), although the curves are still somewhat bowed in the same direction as in Fig. 1. The implication is that in the deteriorated powder, part of the lysine which the free amino-values of the intact protein show to have combined, presumably with sugar, is still available to the rat, just as it is after enzymic hydrolysis to the bacteria used in the microbiological assay.

It appears from Figs. 1 and 2 that the addition of lysine to a deteriorated powder to the extent of rather less than half of the original lysine content of the fresh powder should restore the dietary deficiency of this amino-acid, although it may not raise the content of free lysine quite to its original level. The experiment in which 37° C. 60-day gas-stored H powder was supplemented with 1·25% of lysine equivalent to *c.* 47% of the original content of this acid, showed that the biological value was fully restored, and the protein efficiency greatly increased, although still below the level of the control, largely as a result of a reduced digestibility of the deteriorated protein (Part IV, Exp. 7). Lysine deficiency, therefore, appears to be the main biological defect of the proteins of gas-stored, deteriorated powders of high moisture content. This is in agreement with the conclusion reached from the chemical results that the protein-sugar reaction is largely responsible for other forms of deterioration in this powder.

In Fig. 1 the points for M powder at 37° C. (182 days, air) and H powder at 37° C. (60 days, 'extra air') lie off the curves in a direction which suggests a greater loss of nutritive value than would be expected from the fall in amino-nitrogen (lysine) alone. This would indicate that some additional factor has depressed the nutritive value of these powders. The biological tests (Part IV, Exp. 8) show that the addition of histidine, and possibly of arginine further improves the protein efficiency of deteriorated 'extra air' powder which has already been supplemented with lysine. The effect of supplementing with methionine was not investigated. The results obtained by the microbiological assay method (Part V) substantiate the conclusion that reduced availability of lysine is the major change undergone by the protein in the stored powders, and suggest loss of histidine, arginine and methionine as probable secondary factors. There is thus considerable evidence that in air-stored powder at least, and particularly when storage has been prolonged, changes occur in amino-acids other than lysine.

True digestibility does not appear to be so directly linked with the inactivation of lysine as the biological value, although it is obviously depressed in some way by storage, particularly at high temperatures. Support to this view is given by the behaviour of the M powder, the point for which lies badly off the true digestibility/amino-nitrogen curve (Fig. 1). Further work on this problem is in progress.

Protein deterioration on storage has previously been observed by chemical or biological methods in wheat and wheat flour (4), in soya beans (5, 6), in maize (5, 7), in egg white (8) and, since completion of the present work, in milk powder (9). Destruction of lysine by heat has been observed in milk (10), casein (11) and oat protein (12). With casein and oat protein some of the lysine was rendered unavailable to enzymic digestion, although it could still be released by acid (12, 13).

The analogy between these latter results on the inactivation of lysine by heat and those now reported for storage is striking. There is a discrepancy, however, in that 'pure' proteins such as casein, as well as foods containing both protein and carbohydrate, show inactivation of lysine by heat while, under the conditions of storage employed in the

present work on milk powder the presence of carbohydrate seems to be essential. Whether or not changes leading to the inactivation of lysine can occur in the absence of sugar under more severe storage conditions is not known, and requires investigation. A mechanism whereby the free ϵ-amino-groups of lysine might link up with other parts of the protein molecule, e.g. with free carboxyl groups, is obviously possible during prolonged storage as it is under the influence of heating at a high temperature for a short time.

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APPENDIX

In view of the rapid deterioration in the nutritive value of the proteins which occurred on storage of dried skim milk of high moisture content (cf. Part IV, p. 347) the conditions were further defined under which milk of medium moisture content could be kept for long periods without injury to its proteins.

Samples of M powder (5% moisture) were, therefore, examined after storage for $26\frac{1}{2}$ months at 28·5° C. in air and in nitrogen, and at 20° C. in air. No changes in biological value were observed, while the slight lowering in true digestibility from 93·6 to 91·9 and 92·0 for gas- and air-pack stored at 28·5° C., and to 92·5 for air-pack stored at 20° C. was without statistical significance. It has already been shown (Part IV, Exp. 5) that storage of this milk at 37° C. for 6 months caused a slight but significant decrease in the true digestibility but that no lowering of the biological value of the proteins occurred.

Chemical changes in the powders stored for $26\frac{1}{2}$ months were, in general, very small, the free amino-nitrogen content showing a slight fall consistent with the values given in Fig. 17, Part III and Fig. 1, Part VI.

It follows that powders containing not more than 5% moisture can be stored in air- or gas-pack at temperatures up to 28·5° C. (83° F.) for at least 2 years without suffering detectable loss in the nutritive value of their proteins, though with a serious loss of flavour in the former and a less-marked loss in the latter (cf. Figs. 1 and 2, Part III). Furthermore, it seems likely that under all conditions of storage skim-milk powder will become unpalatable before it suffers any appreciable loss in the nutritive value of its proteins.

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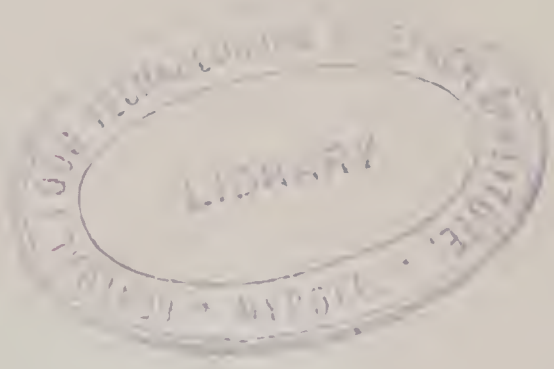
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